GLUCOSAMINE RESISTANCE IN THE YEAST *SACCHAROMYCES CERIVISAE*

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SUMMARY

By using glucosamine resistant mutants of Saccharomyces cerivisae an attempt was made to discover the mechanisms which cause glucose repression and/or the Crabtree effect. The strains used are 4B2, GR6, $10P_3r$, GR81 and GR108. 4B2 is a wild type yeast while the others are its mutants. To characterize the biochemical reactions which made these mutants resistant to glucosamine poisoning the following experiments were done: 1. growth and respiration; 2. transport of sugars; 3. effect of inorganic phosphate (Pi); 4. Hexokinase; 5. In vivo phosphorylation.

From the above experiments the following conclusions may be drawn:

(i) GR6 and $10P_3r$ have normal respiratory and fermentative pathways. These mutants are resistant to glucosamine poisoning due to a slow rate of sugar transport which is due to change in the cell membrane.

(ii) GR81 has a normal respiratory pathway. The slow growth on fermentable carbon sources indicates that in GR81 the lesion is in or associated with the glycolytic pathway. The lower rate of sugar transport may be due to a change in energy metabolism. The in vivo

phosphorylation rate indicates that in GR81 facilitated diffusion is the dominant transport mechanism.

(iii) GR108 has a normal glycolytic pathway but the respiratory pathway is abnormal. The slow rate of sugar transport is due to a change in energy metabolism. The lower percentage of *in vivo* phosphorylation is probably due to a lowered availability of ATP because of the mitochondrial lesion.

In all mutants resistance to glucosamine poisoning is due to a lower rate of utilization of ATP, which is caused by various mechanisms (see above), making less ADP available for phosphorylation via ATP synthase which utilizes inorganic phosphate. Because of the lower utilization of Pi, the concentration of intra-mitochondrial Pi does not go down thus protecting mutants from glucosamine poisoning.

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INTRODUCTION

All living things have an ability to adapt their metabolism to changes in their environment. Such an adaptation, that is a regulation of metabolism is present in micro-organisms, in which the environment causes a change in metabolism, which then leads to synthetic responses mediated by genetic control mechanisms (Ephrussi, Slonimski, Yotsuyangi, and Travlitzki, 1956; Jacob and Monod, 1961)

The facultative anaerobic yeast Saccharomyces cerivisae is a micro-organism which represents an ideal system for the study of mitochondriogenesis, an adaptive process. This yeast was used in this study because it is a facultative anaerobe. This means that it can grow in both aerobic and anaerobic culture conditions with equal facility.

The metabolic needs of the cell are very different under these two atmospheric conditions. Under anaerobic conditions growth is dependent on fermentation of sugars which produces ethanol and carbon dioxide as end products. When yeast is growing aerobically on a nonfermentable carbon source the metabolic energy is derived from respiration. Nonfermentable or respirable carbon sources such as ethanol or glycerol are oxidised via the tricarboxylic

acid or Kreb's cycle to produce carbon dioxide and reduced nicotinamide adenine nucleotides (NADH, NADPH). The NADH is then oxidised via the respiratory chain to produce the high energy intermediate adenosine triphosphate (ATP) with molecular oxygen as electron acceptor. Thus the yeast uses the respiratory chain under conditions when the energy derived from fermentation is insufficient for growth and an alternative respirable energy source is available.

There are two major physiological control phenomena which exist in yeast cells (Linnane and Haslam, 1970). The first is the catabolite repression of mitochondrial formation induced by growth of the organism on fermentable substrates. The other is a requirement for oxygen for the formation of normal, functional mitochondria. Under anaerobic conditions yeast cells develop little or no respiratory activity or mitochondria (Ephrussi, 1950; Slonimski, 1953) and their exposure to oxygen induces the synthesis of mitochondria (Slonimski, 1953). If cells are grown aerobically on a high concentration of glucose, they develop very little respiratory activity and contain only reduced amounts of mitochondrial enzymes. This phenomenon is called catabolite or glucose repression (Schatz, Haslbrunner and Tuppy, 1964). This effect is of a transitory nature; i.e., when glucose is exhausted respiration becomes derepressed (Utter, Duell and Bernofsky, 1968; Jayaraman, Cotman, Mahler and Sharp, 1966).

In glycerol grown yeast cells the addition of glucose causes inhibition of respiration (Belitzer, 1936; Loomis and Lipman, 1948) and the inhibition of respiration is termed the Crabtree effect. This inhibition is observed in parallel to glucose repression. The Crabtree effect by glucose is also of a transitory nature. D(+) Glucosamine also induces the Crabtree effect (Letansky, 1968) but inhibition is of an irreversible nature because glucosamine is not metabolized after glucosamine-6-phosphate is formed. Glucosamine also inhibits growth of glycerol grown yeast cells.

It was hoped that the isolation of glucosamine resistant mutants might help us discover how glucose repression controls mitochondriogenesis and whether or not there is some or no connection between glucose repression and the Crabtree effect.

A brief review of the relevant literature is found below.

CHAPTER 1

REVIEW OF THE LITERATURE

Crabtree Effect

The phenomenon of glucose induced respiratory inhibition was first observed in slices of solid carcomas and carcinomas (Crabtree, 1929) and hence it is called the Crabtree effect. The inhibition is often called the reversed or inverted Pasteur effect (The Pasteur effect is the inhibition of glycolysis by respiration which was first reported by Pasteur in 1861). Although the Crabtree effect has been demonstrated in yeast cells (Belitzer, 1936; Loomis and Lipman, 1948; DeDeken, 1966) most of the evidence for the Crabtree effect comes from mammalian systems. Respiratory inhibition can be induced by glucose, fructose, mannose (Brin and McKee, 1956) 2 deoxy glucose (Ibsen, Coe and McKee, 1958) and glucosamine (Scholefield, 1958).

Belitzer (1936) and Loomis and Lipman (1948) suggested that competition between glycolysis and respiration for common intermediates might be the basis of the Crabtree effect. In the presence of an uncoupler DNP (2,4 dinitrophenol) the effect was released showing that there might be a competition for inorganic phosphate (Pi) or adenine nucleotides between glycolysis and respiration.

Besides the evidence from the use of uncouplers further evidence for the involvement of Pi in the inhibition of respiration has also been given, e.g., (a) the Pi level is lowered during glycolysis (Acs and Straub, 1954; Racker, 1956; Hess and Chance, 1959); (b) increased amounts of extracellular Pi reduced the Crabtree effect in mouse leukemia cells (Brin and McKee, 1956);
(c) limitation of Pi caused respiratory inhibition in reconstructed in vitro systems (Gatt and Racker, 1959).

Evidence has also been presented showing that Pi is not the only factor involved in the Crabtree effect. Increasing the Pi level of the medium can also increase the Crabtree effect (Bloch-Frankenthol and Ram, 1959) in ascites tumor cells.

Adenine dinucleotide (ADP) might also be a limiting factor, rather than Pi, and supporting evidence has also been reported, e.g., (a) shortly after glucose addition there is a decrease in the endogenous ADP level (Ibsen, et al., 1958); (b) in isolated mitochondria ADP controls respiration more effectively than Pi (Chance and Hess, 1959); (c) limitation of ADP caused inhibition of respiration in reconstructed systems (Gatt and Racker, 1959); (d) the ratio of ATP to ADP (ATP/ADP) in glycolysing cells is usually lower than the ratio in non glycolysing controls (Hess and Chance, 1961).

In yeast cells Polakis and Bartley (1966) observed a decreased level of ADP during glucose repression and they

suggested that ADP levels control the interaction between respiratory and fermentative pathways. Ball and Tustanoff (1970) observed no ADP change during derepression (release of glucose repression) and they suggested that ADP control of respiration and fermentation is unlikely.

The effect of ADP and Pi on glycolysis, rather than on the interaction between glycolysis and respiration, has also been studied. Working on yeast cells Lynen (1959) suggested that ADP control of glycolysis is unlikely as long as ATP is available for hexose phosphorylation. Several investigators have shown that aerobic as well as anaerobic glycolysis in ascites cells is primarily regulated by the concentration of Pi (Wu and Racker, 1959; Ibsen, et al., 1960; Uyeda and Racker, 1965). Also phosphate stimulates hexokinase and phosphofructokinase activity even in the presence of inhibiting amounts of their products (Wu, 1965). These observations lend further credence to the idea that inorganic phosphate controls or limits glycolysis.

The reverse of the Crabtree effect, the Pasteur effect, has been of great interest in yeast cells. The factors thought to be responsible for the Pasteur effect are also key factors in the Crabtree effect. Discussion of the Pasteur effect is of great significance for the explanation of the Crabtree effect. Many theories have been presented to explain the Pasteur effect. One of them is the depletion of inorganic phosphate due to coupled

phosphorylation under aerobic conditions (Lynen, 1941; Johnson, 1941).

Lynen, Hartman, Netter and Schuegraf (1959) working on yeast cells and Racker (1965) in ascites cells proposed an important role for inorganic phosphate at three enzyme levels.

(i) At glyceraldehyde-3-phosphate dehydrogenase,

(ii) Counteracting the inhibition of phosphofructokinase by ATP,

(iii) Counteracting the inhibition of hexokinase by glucose-6-phosphate.

The competition for phosphate and adenine nucleotides, superimposed on the multiple controls of hexokinase and phosphofructokinase is the accepted explanation for the Pasteur effect, although according to Racker (1974) the control mechanisms in different cell types are still very complex and poorly understood.

The explanation of the Crabtree effect is analogous to that of the Pasteur effect, though allosteric mechanisms are apparently not involved. ATP, ADP, and Pi are key factors in the Crabtree effect. Glycolysis and the respiratory chain both compete for ADP and Pi and when glycolysis wins the result is the Crabtree effect (Krebs, 1972).

When an excess of a metabolizable sugar, e.g., glucose, is added to yeast cells, the transport of sugar increases inside the cells (Fig. 1). The sugar might be phosphorylated

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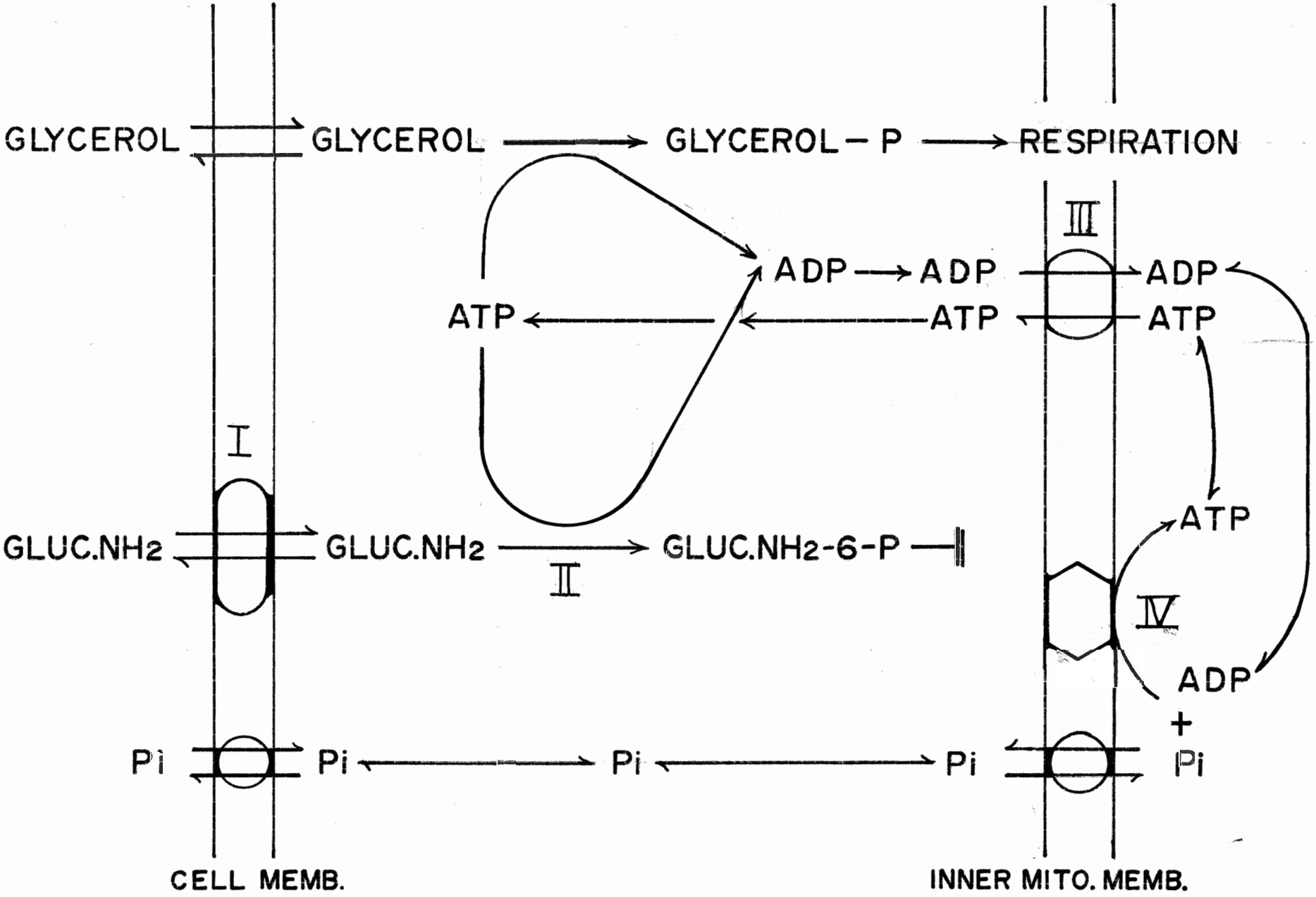
Fig. 1: Possible Sites for Glucosamine Resistance in Yeast Cells.

Site I	Sugar Carrier
Site II	Hexokinase
Site III	Translocase
Site IV	ATP - Synthase

A POSSIBLE MECHANISM FOR GLUCOSAMINE POISONING IN YEAST



during transport (Van Steveninck, 1968 and 1969) or inside cells via the hexokinase reaction (Cirillo, 1962; Kotyk, 1973) using up ATP (Fig. 1, Site II). The utilization of ATP will increase the cytoplasmic ADP level. The lowered ATP level will stimulate the translocation system (Fig. 1, Site III) transporting ADP into the mitochondrion. This ADP will stimulate the ATP ase (Fig. 1, Site IV) producing an acceleration of respiration (Ibsen, 1961) and utilization of inorganic phosphate (Pi). A continued demand for ATP will lead to more formation of ATP via ATP ase with a decrease in Pi level. Depletion of Pi also occurs when glyceraldehyde-3-phosphate dehydrogenase uses Pi, thus increasing the mitochondrial Pi depletion, which causes a subsequent repression of respiration. Again, in the presence of glucose this repression is transitory because of the subsequent availability of ATP from glycolysis (Ibsen, 1961; Chance and Hess, 1956). It is suggested that when glucosamine is added to the cells similar events occur, but the repression is non-transitory because of the unavailability of ATP from glycolysis. Glucosamine traps the Pi in glucosamine-6-phosphate because glucosamine-6-phosphate is non-metabolisable. Glucosamine-6-phosphate cannot enter glycolysis, because it is not a substrate for glucose phosphate isomerase (Bessel and Thomas, 1973). In carcinoma cells glucosamine-6-PO_4 is utilized in the formation of mucopolysaccharides and glycoproteins (Letnansky, 1968).

In yeast glucosamine-6-phosphate can be utilized in chitin synthesis although this is a minor pathway (Cabib and Keller, 1971).

Catabolite and Glucose Repression

When yeast cells are exposed to a high glucose concentration, the inhibition of respiration results in a decreased rate of mitochondriogenesis. This repression of enzyme synthesis is not transient and can be expressed over periods of hours provided sufficient hexose is present in the medium. This kind of glucose effect is called glucose repression or catabolite repression. The occurence of catabolite repression of respiratory ability was first described by Slonimski (1955) and Ephrussi et al. (1956) and has been further studied by numerous investigators (Polakis and Bartley, 1965; Jayaraman, Cotman, Mahler and Sharp, 1966; Ball and Tustanoff, 1970). Cells grown on slowly fermentable sugars, such as galactose show intermediate respiratory activity (Tustanoff and Bartley, 1964; Ball and Tustanoff, 1971). Glucosamine and 2 deoxy glucose also act as catabolite repressors of the formation of mitochondrial enzymes in yeast cells. The repressed mitochondria are heterogenous in their contents of respiratory enzymes (Schatz, Haslbrunner and Tuppy, 1964).

The release of repression or derepression results when glucose in the medium becomes utilized by glycolysis.

During this time the respiratory enzymes increase greatly in activity and the mitochondria apparently increase in number (Linnane, 1965; Lukins, Jollow, Wallace and Linnane, 1968).

A detailed review of the very large and extensive body of literature which documents the modification of yeast mitochondrial structure and function by glucose repression is beyond the scope of this section. Recent reviews by Linnane and Haslam (1970) and Perlman and Mahler (1974) cover this literature in great detail.

Apart from its repressive effect on mitochondria, catabolite repression is also known to affect the synthesis of enzymes responsible for the hydrolysis of disaccharides in yeast (MacQuillan, Wilderman and Halvorson, 1960; VanWijk, Quwehand and VandenBos, 1969). In bacteria, e.g., E. coli, catabolite repression is correlated with a reduced content of 2 - 3 cyclic adenine mononucleotide (cAMP) within the cells and it has been shown that externally added cAMP is able to overcome repression of inducible enzyme synthesis (Ullman and Monod, 1968; Erron, Arditti and Beckwith, 1971). Experimental results concerning the regulation of the lactose operon in E. coli have shown that cAMP is a prerequisite for the synthesis of the enzymes in this operon, acting at the promotor region (Erron et al., 1971; Perlman, Chen, DeCrombrugghe

and Pastan, 1970). Little is known about the function of cAMP in yeast cells. An increase in cAMP level was observed when yeast was derepressed from glucose repression (VanWijk and Kowijn, 1971) although the work has not been repeated and the methods are suspect because they used a slime mold assay for cAMP. Janki and Tustanoff (Janki, Ph.D. thesis, 1972) observed a decrease in cAMP which was coincident with the exhaustion of glucose or galactose in aerobically grown yeast. They used a more accurate assay (^{14}C immuno-precipitate assay) for cAMP. In Schizosaccharomyces pombe, it was shown that the concentration of intracellular cAMP was lower in glucose repressed cells than in derepressed ones (Megnet and Schalanderer, 1971). Mutants partially resistant to repression show a greater synthesis of catabolite sensitive enzymes and a higher concentration of cAMP (Schlanderer and Dellweg, 1974). It has also been suggested that polyphosphates are involved in catabolite repression by binding the cAMP binding protein, which is necessary for transcription. Except for one unconfirmed report by Fang and Butow (1970) there is no evidence that cAMP is involved in the regulation of mitochondriogenesis in Saccharomyces cerivisae.

Transport

Three kinds of transport mechanisms have been postulated by which hexoses may be taken up by cells (Lehninger, 1975).

(i) Simple Diffusion - requires no specific carrier or metabolic energy.

(ii) Facilitated Diffusion - in this type specificity plays an important role due to carrier protein whose function is to facilitate transport through the cellular membrane although again, no energy is required.

(iii) Active transport - requires metabolic energy. Like facilitated diffusion the function of a specific carrier protein is assumed which occurs in two forms, energised and non-energised. Metabolite uptake in yeast does not proceed to any significant extent by simple diffusion (Kotyk, 1974).

Kinetic studies involving single and paired hexoses have firmly established that the transport of these sugars is carrier mediated (Scharff and Kremer, 1962; Cirillo, 1962). In all cases hexose transport obeys Michaelis-Menton kinetics. Glucosamine and sorbose uptake is mediated by the same carrier and is competitively inhibited by glucose (Cirillo, 1961; Scharff and Kremer, 1962). Further evidence for carrier mediated sugar transport has been obtained from studies with induced and non-induced galactose cells (Haskovec and Kotyk, 1969; Kuo and Cirillo, 1970; VanSteveninck, 1972).

Although there is a general agreement that sugar transport requires carrier mediation there is disagreement as to whether or not metabolism is directly involved in the

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Fig. 2: Sugar Transport Mechanisms.

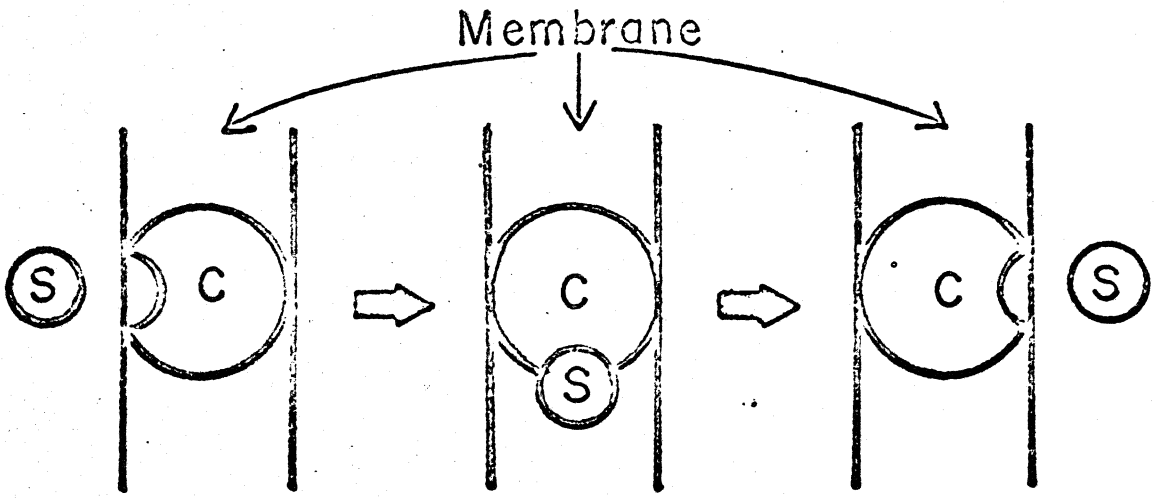
A - Facilitated diffusion
(Cirillo, Kotyk, Van Steveninck)

B - Active Transport
(Van Steveninck)

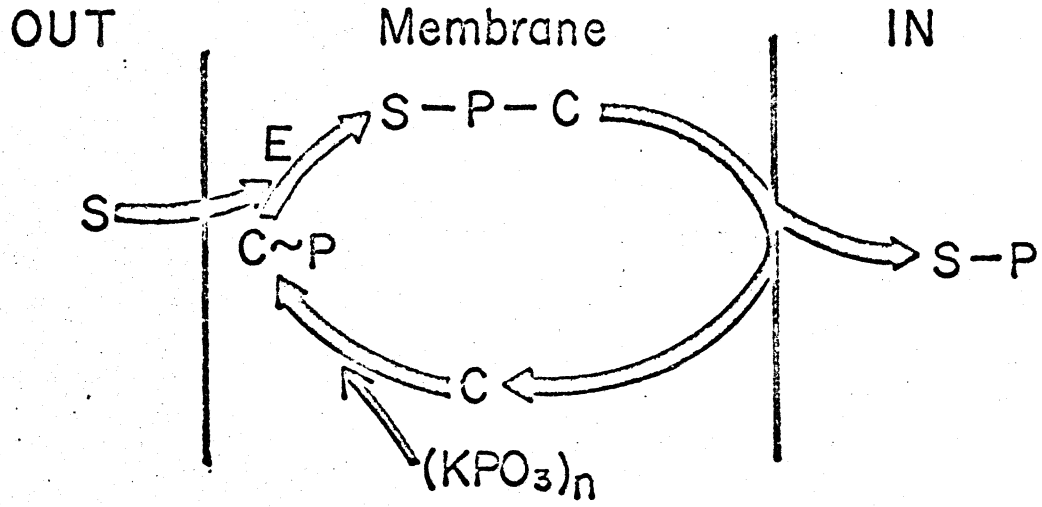
C - Facilitated diffusion with metabolic draft
(Cirillo)

S = Sugar, C = Carrier, E = Permease, S-P = Sugar phosphate, C~P = Energised carrier.

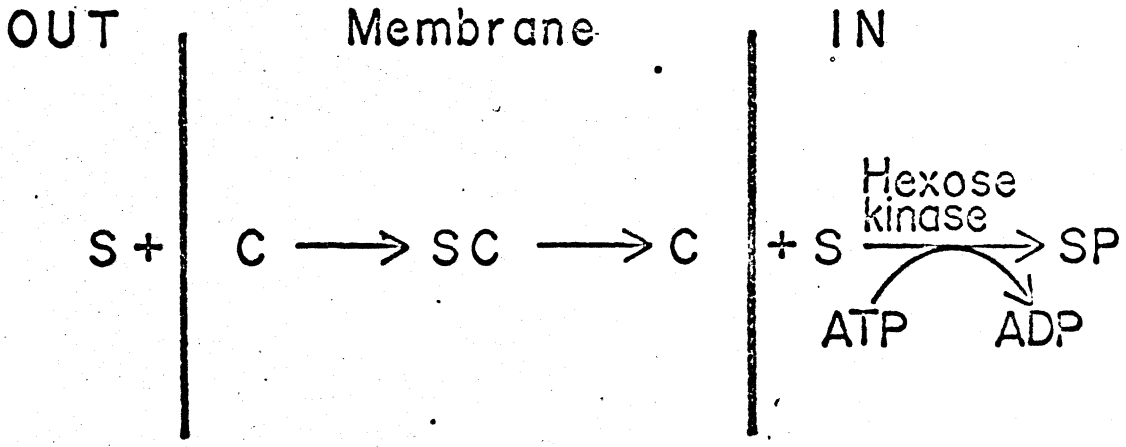
(Adopted from Blair, 1975)


Membrane
S
C
C
S
C
S

A


OUT
Membrane
IN
S
E
S-P-C
C~P
S-P
C
(KPO3)n

B


OUT
Membrane
IN
S +
C
SC
C
+ S
Hexose kinase
SP
ATP
ADP

C

process. VanSteveninck and coworkers suggest that there are two mechanisms of hexokinase transport; facilitated diffusion (Fig. 2A) and an energy dependent, phosphorylating transport system (Fig. 2B).

Cirillo and coworkers (1962 and 1970) and Kotyk (1973) believe that sugar transport proceeds only by facilitated diffusion and that phosphorylation takes place inside the cell by the action of soluble kinases (Fig. 2C).

The basic observations about the phosphorylation involvement during transport were made by VanSteveninck and Rothstein (1965). They showed that glucose normally is transported into yeast cells by a system of high affinity and relatively high specificity. In the presence of the metabolic inhibitor iodoacetate, glucose entered by a broader specificity facilitated diffusion system which also transported galactose and sorbose. When glucose is transported by the high affinity system a drop in the binding of nickel and cobalt ions by the cell surface is observed (VanSteveninck and Rothstein, 1965; VanSteveninck, 1966), and transport is inhibited by uranyl ions (UO_2^{2+}) at relatively low concentrations. When facilitated diffusion is occuring however, there is no change in the binding of nickel or cobalt ions and only high concentrations of UO_2^{2+} inhibit transport. It had been shown earlier that nickel, cobalt and uranyl ions at low concentrations bind

to polyphosphate groups at the cell surface (Rothstein, 1954; VanSteveninck and Booij, 1964).

VanSteveninck (1969) also showed in iodoacetate poisoned cells that there is a rapid uptake of about 5 μmole of glucose per g of yeast. No free glucose could be recovered from the cells and it was found that the transported sugar had been phosphorylated by polyphosphates because the ATP concentration was too low (less than 0.1 μmole/gm of yeast) to account for the phosphorylation of glucose via the hexokinase reaction. Similar results were obtained with 2-deoxy D-glucose (VanSteveninck, 1968). The source of phosphate is believed to be polyphosphate formed in the cell from ATP.

Kotyk and coworkers believe that sugar transport into yeast cells proceeds only by facilitated diffusion (Kotyk, 1973). According to them there are three monosaccharide transport systems in yeast (Jennings, 1974). Two are constitutive, one being a low specificity transport system and the other having higher specificity for glucose type sugars. The higher specificity system is believed to be inhibited by uranyl ions while the low specificity system is not (Kotyk, 1973). There is a third, inducible, system which transports D-arabinose, D-galactose, D-glucose, D-ribulose and D-xylose and functions at low concentrations of these sugars. Kotyk does not believe the transmembrane phosphorylation hypothesis of VanSteveninck because of his taking the pulse-label

measurements at too long an interval after the pulse (Kotyk, 1973).

Cirillo bases his rejection of the transmembrane phosphorylation hypothesis on the facts that the membrane is permeable to sugars which are not phosphorylated and that nonmetabolized sugars are transported via a nonconcentrative process sensitive to uranyl ions but insensitive to metabolic inhibitors (Cirillo, 1962). He further found that uptake of nonmetabolized sugars is competitively inhibited by the metabolized sugars and that metabolized sugars can induce the uphill efflux of nonmetabolized ones in cells previously equilibriated with the nonmetabolized sugars (Cirillo, 1968). He also concluded that metabolized and nonmetabolized sugars are transported via the same carrier, the difference in uptake rates being due to the different affinities of the sugars for the carriers.

A recent study has shown that different experimental conditions will produce different kinetics for the same transport system (Serrano and Delafuente, 1974). They obtained two apparent Km's and Vmax's for cells treated with icdoacetic acid and uranyl ions. This provides new evidence concerning the existence of two forms or two states of the carrier which, in the VanSteveninck model, co-exist *in vivo*.

Kuo and Cirillo (1970) proposed a compromise scheme of the facilitated diffusion and active transport

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Fig. 3: An Integration of Transport Models in Baker's Yeast.

The carrier mediated, facilitated diffusion pathway is indicated by solid arrows, and the active transport phosphorylating pathway by double line arrows.

S and SP = free and phosphorylated sugar

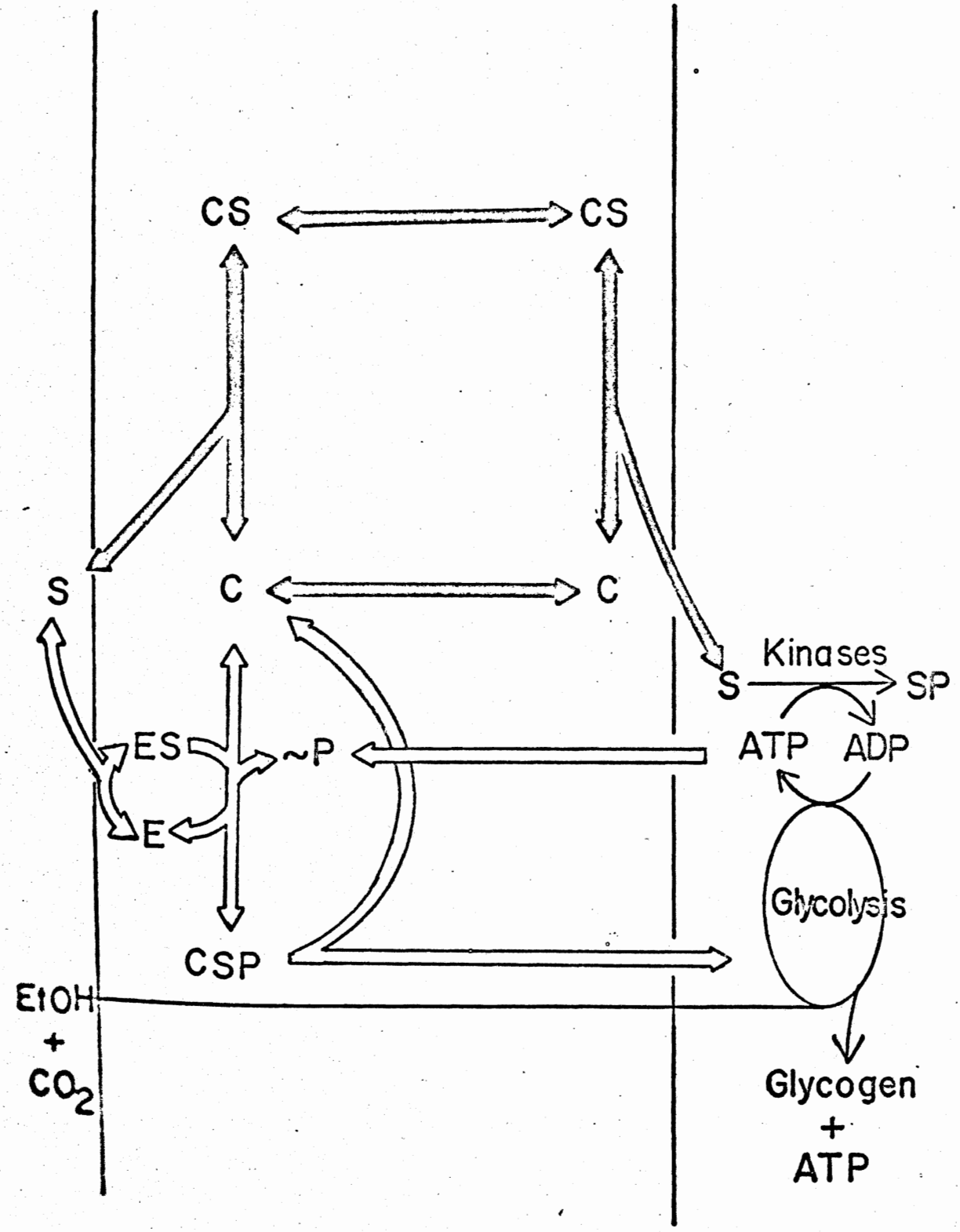
C and CS = free and loaded carrier

E and ES = Permease and Permease-Sugar complex

X~P = phosphate donor

CSP = permease catalyzed carrier - Sugar - phosphate complex

(from Cirillo, 1970)


CS
CS
S
C
C
Kinases
S
SP
ATP
ADP
ES
~P
E
Glycolysis
CSP
EtOH
+
CO_2
Glycogen
+
ATP

mechanisms (Fig. 3).

It is easily visualized that the S $\rightarrow$ SP hexose-kinase mediated reaction has a higher affinity for ATP than the ATP $\sim$ P reaction. At low ATP concentration transport should proceed by fascilitated diffusion followed by phosphorylation. At high ATP concentrations transport should proceed via the active transport mechanism. Under intermediate condition the two systems may coexist. The net effect is that the observed Km will be a function of the cells energy metabolism (Cirillo, 1961).

From the above study it is clear that glucose and its analogues, 2 deoxy glucose and glucosamine are transported by the same carrier and that ATP is required to effect rapid transport.

Adenine Nucleotide Translocating System in Yeast

Mitochondria are generally considered to be cellular orgarelles specialized for aerobic production of ATP. The system translocating the adenine nucleotides between the mitochondrion and the cytoplasm has been a target for recent investigations of mitochondria because of its possible involvement in energy conservation and in the control of cell growth (Subik, Kolarov and Kovac, 1972). Yeast mutants which are affected in their mitochondrial structure and function do not entirely lack mitochondrial structures (Subik et al., 1972). Non-respiring anaerobically grown cells exhibit transfer reactions (ATPase) and

translocation of ions and adenine nucleotides (Groot, Kovac and Schatz, 1971; Kolarov, Subik and Kovac, 1972). The mitochondrial adenine nucleotide translocation system remains in ρ^- (respiratory deficient) mutant cells (Subik, Kolarov and Kovac, 1974; Groot, Out and Souverijin, 1975). In the ρ^- mutants studied by Borst (1972) mitochondrial DNA was absent so the enzyme systems still present must be coded for in the nucleus, and synthesised on cytoplasmic ribosomes (Kolarov and Klinegenberg, 1974). Also all bongkrekic acid (inhibitor of translocating system) resistant mutants so far isolated are nuclear (Perkins, Haslam and Linnane, 1972) not mitochondrial mutants.

The adenine nucleotide transport system of the mitochondrion involves the exchange between extra- and intramitochondrial ADP and ATP, a reaction which is essential to the transfer of energy from oxidative phosphorylation to extramitochondrial processes. The ADP, ATP carrier is the exclusive link between inner- and extramitochondrial Pi transfer reactions (Heldt and Klingenberg, 1965). This is based on the specificity for ADP and ATP which excludes AMP and all other nucleotides (Duee and Vignais, 1969; Winkler, Bygrave and Lehninger, 1968). It is located in the inner mitochondrial membrane (Klingenberg and Pfaff, 1965). The ADP, ATP transport represents an exchange of ADP or ATP against the intramitochondrial nucleotide pool such that there is no net accumulation in the

mitochondria (Klingenberg and Pfaff, 1965). The exchange is limited by the size of the endogenous pool of ADP + ATP which can change on account of the interconversion of endogenous AMP, ADP, and ATP (See below). The exchange between ADP and ATP is basically non-energy dependent (Pfaff, Heldt and Klingenberg, 1969; Klingenber, 1975). By the use of inhibitors, e.g., Atractylate (ATR), Bongkrekic acid (BKA) and Carboxy atractylate (CAT), it has been established that at the binding site there are three positive charges and that carrier sites are asymetrical (Weidemann, Erdell and Klingenberg, 1970; Klingenberg and Bucholz, 1973) with respect to the inside and outside of the membrane.

Mutation of the adenine nucleotide translocating system in glucosamine resistant mutants could cause a lower rate of transport of ATP from mitochondria to the cytoplasm. In return less ADP will be available in mitochondria for phosphorylation and the Pi concentration would not go down so fast thus protecting these mutants from glucosamine poisoning (See Crabtree effect, above).

The adenine nucleotide transporting system of mitochondria does not transport nucleotides other than ATP and ADP, the conversion of ADP and ATP to other nucleotides, e.g., AMP or cAMP might disturb the balance of ADP and ATP inside or outside of the mitochondria. So a discussion of the enzymes which are responsible for interconversion of nucleotides is necessary.

Adenine Nucleotide Interconversion

The intracellular compartmentation of ATP is an important feature in cellular regulation of metabolism. The role of ATP and ADP in the Crabtree and Pasteur effects and of cAMP on catabolite repression has been discussed in earlier sections. As these nucleotides play a very important role in yeast metabolism it will be a good idea to discuss the interconversion of these nucleotides.

The enzymes of glycolysis which utilize and produce ATP are given below.

(i) Hexokinase

$$\text{Glucose} + \text{ATP} \longrightarrow \text{G-6-P} + \text{ADP}$$

(ii) Phosphofructo kinase

$$\text{Fructose-6-P} + \text{ATP} \longrightarrow \text{F-1-6-di-P} + \text{ADP}$$

(iii) Phosphoglycerate kinase

$$\begin{array}{c} \text{3 - Phosphoglyceroyl phosphate} + \text{ADP} \\ \Downarrow\Uparrow \\ \text{3 Phosphoglycerate} + \text{ATP} \end{array}$$

(iv) Pyruvate kinase

$$\text{phosphoenolypyruvate} + \text{ADP} \rightleftharpoons \text{Pyruvate} + \text{ATP}$$

The ATP is also produced in mitochondria by coupled oxidative phosphorylation. The enzyme is called an **ATPase.**

$$\text{ADP} + \text{Pi} \rightleftharpoons \text{ATP}$$

Besides the above enzymes of glycolysis and respiration there are some other enzymes which are involved in the interconversion of adenine nucleotides which are given below.

(i) Adenylate Kinase or Myokinase (Extramitochondrial enzyme)

$$2\ ADP \rightleftharpoons ATP + AMP$$

(ii) Adenyl Cyclase

$$ATP \rightleftharpoons 2'3'\ cAMP + P\text{-}P$$

(iii) Phosphodiesterase

$$cAMP + H_2O \rightleftharpoons AMP \text{ (adenosine 5'phosphate)}$$

(iv) GTP-AMP phosphate transferase present in the matrix of the mitochondrion

$$GTP + AMP \rightleftharpoons GDP + ADP$$

(v) Monophosphate kinases which are present in the inter-membrane space, e.g., AMP kinase

$$AMP + ATP \rightleftharpoons ADP + ADP$$

(vi) Other nucloside phosphate kinases involved in inter-conversion of nucleotide phosphates, e.g., nucleotide diphosphate kinase

$$GDP + ATP \rightleftharpoons ADP + GTP$$

Mutation in any of these enzymes could upset the adenine nucleotide balance or energy charge of cells which could in turn affect sugar phosphorylation (Ball and Atkinson, 1975) and cause resistance. As I mentioned earlier (Introduction) glucosamine has a limited metabolism in yeast cells therefore the enzymes of the glycolytic pathway, except hexokinase, cannot be responsible for glucosamine poisoning. There is also no confirmed evidence about cAMP involvement in catabolite repression in yeast cells so the role of adenyl

cyclase and phosphodiestrase in glucose repression remains in doubt.

CHAPTER 2

MATERIALS AND METHODS

Saccharomyces cerevisae, strains 4B2 and 4BL, and four glucosamine resistant mutants were used for this study. The glucosamine resistant mutants were produced by EMS or UV mutagenesis of strains 4B2 and 4BL.

The parental strains 4B2 and 4BL were derived from D 587-413 and D 585-11C respectively. These strains were a gift of Dr. Fred Sherman, Department of Radiation Biology, Rochester University, New York, U.S.A. The genotypes of the parental strains and glucosamine resistant (GR) mutants are as follows:

Strain	Nuclear	Mitochondrial	Cytoplasmic
4B2	α, his1	[rho$^+$]	[KIL-K]
4BL	α, lys1	[rho$^+$]	[KIL-K]
GR6	α, his1	[rho$^+$]	[KIL-K,GGM-r]
GR10	α, his1	[rho$^+$]	[KIL-K,GGM-r]
GR81	α, lys1,gay1	[rho$^+$]	[KIL-K]
GR108	α, lys1,gay2	[rho$^+$]	[KIL-K]

The gay1 and gay2 are nuclear loci (Ball, Elliot and Wong, 1976). Glucosamine resistance in GR6 and GR10 (derivative $10p_3r$) is non-nuclear as indicated by GGM.

All strains were maintained aerobically on agar slopes containing 1% (w/v) Bacto yeast extract, 2% (w/v) Bacto peptone, 3% (w/v) dextrose, and 2% (w/v) Bacto agar. The yeast slopes were stored at 2^{o} after growing for 24 hours at 30^{o}.

Media

(i) YPD - Contains:

3% (w/v) glucose

2% (w/v) Bacto peptone

1% (w/v) Bacto yeast

(ii) YPG - Ingredients are as for YPD, except 3% v/v) glycerol was used instead of glucose.

(iii) YP gal - Same as YPD with galactose instead of glucose.

(iv) YP mal - Same as YPD with maltose used instead of glucose.

Growth of Cells

YPD, YPG, YPgal and YPmal liquid media were used for growing cells. The yeast inoculum was prepared by washing the cells from 24 hour slopes with sterile distilled water. Sufficient inoculum (determined by absorbance at 415 nm) was added to liquid media to give reproducible lag phases as appropriate. For glucose and maltose media 1 ml (O.D. 415 = 0.15) of yeast suspension was used for 100 ml of medium. For glycerol and galactose 1 ml (O.D. 415 of 1/10 dilution = 0.20) of yeast suspension was used for 100 ml of liquid medium. Cells were grown aerobically in 500 ml conical

flasks which were aerated by vigorous shaking at 300 rpm at 30°. Aliquots were taken at various intervals for determination of oxygen uptake, absorbance and protein estimation.

Harvesting the Yeast Cells

Yeast cells were collected at different time periods, transferred into ice-cold centrifuge tubes and centrifuged at 2,000 rpm for 10 minutes in a refrigerated centrifuge. The resulting pellet was washed twice with ice-cold distilled water. If enzyme assays were to be carried out, the cells were stored at -20° until used.

Protein Determination

Known aliquots (5.0 ml) of the growing cells were centrifuged at top speed in a bench top IEC clinical centrifuge for three minutes. The supernatant was discarded and the pellet washed twice with 0.5N perchloric acid. The resultant pellet was resuspended in a known volume (2.0 ml) of 2.0 N NaOH and incubated for 24 hours at room temperature. The NaOH suspensions were then centrifuged and known aliquots were taken for protein assay which was determined by Lowry, Rosenbrough, Farr and Randall (1951) method. Standard protein was Crystalline Bovine serum albumin.

Measurement of Respiratory Ability

[i] Polarographic: At various intervals during the growth period 5 ml aliquots were taken from the culture and

transferred to the chamber of an oxygen monitor (Yellowspring Instrument Co., Yellowspring, Ohio; Model 53) maintained at 30°. Cells were then aerated for approximately 1 minute at a constant rate. Oxygen consumption of the whole cells was monitored polarographically using a Clark type electrode for 3 - 5 minutes at 30°. These measurements gave the respiratory capacity of yeast cells at that time. Very actively respiring cells were diluted with the supernatant obtained from the simultaneous samples taken for the protein estimations.

[ii] Gilson Respirometer: Respiratory rates were also determined using a Gilson respirometer when the effect of glucosamine, glucose, deoxy galactose, galactosamine, and/or inorganic phosphate were to be determined on growing cells. Mannometer vessels were prepared as follows:

The main compartment contained yeast cells 2.4 ml (O.D. 415 of 1/10 dilution = 0.12) in YPG, $\pm$ 0.1 ml of 2M KH_2PO_4. The side arm contained 0.2 ml of either glucose, glucosamine, galactosamine or deoxy galactose.

The concentration of glucose or glucosamine solution was 1.5%. The concentration of deoxy galactose was 6.0%. The center well contained 0.1 ml of 6M KOH to absorb CO_2. Flasks were equillibriated at 30° for 10 minutes before the test was started by tipping the side arm into the main compartment. Readings were taken every five or ten minutes for 120 minutes. This allowed constant monitoring of the oxygen consumption over relatively long time periods.

Sugar Transport Experiments

The glucose, glucosamine and sorbose uptake of glycerol or glucose grown cells was done with mid log and stationary phase cells. These cells were washed twice with cold distilled water and resuspended in distilled water for transport experiments. Glycerol grown unwashed cells were also used for some transport studies. Different concentrations of glucose, glucosamine and sorbose were used for determination of Km and Vmax. The radioactive substrate was also increased with increase in substrate to make the specific activity constant (Ratio of ^{14}C substrate/total substrate).

For YPG grown cells, tested in YPG medium 0.5 ml samples were taken onto 25 mm. membrane filters (Millipore Membrane AAWP0, 2500, Millipore Corp., Bedford, Massachusetts, U.S.A.) or 23 mm. glass fibre filters (r.a. glass fiber filter, grade 934 AH, Whatman Inc., Clifton, New Jersey, U.S.A.) and washed with 5 ml distilled water containing the same substrate concentration as was used for the transport experiment. For experiments with washed yeast cells 0.1 ml samples, washed with 2.0 ml of the appropriate sugar solution were used. The filter was then transferred to a scintillation vial containing 1 ml anhydrous methanol. Then scintillation fluid (10 ml of PCS:xylene mixture, 1:2 ratio) was added. The radio-activity was measured in a Packard Tri Carb liquid scintillation counter. Counts per minute were converted to dpm using the internal channels ratio method.

In Vivo Phosphorylation

At various intervals 0.5 ml samples from transport experiments were taken onto glass fibre filters and washed with 5 ml distilled water containing the same substrate concentration as was used for the transport experiment. The filter was then transferred to a tube containing 1 ml of ethanol (80% ethanol containing 5 mg/ml of glucosamine and glucosamine-6- phosphate as cold carrier). The sample was then incubated at 80° for 10 minutes and then cooled in ice (Chapman, Fall and Atkinson, 1971). The denatured protein was removed by centrifugation and the supernatant was used for chromatography (see below). The in vivo method separates phosphorylated glucosamine and its possible derivatives from glucosamine.

Chromatography

Ethanol extracts were chromatographed to separate glucosamine from glucosamine-6- phosphate. Known aliquots of supernatant were spotted onto thin layer cellulose, chromatographic sheets (Pre-coated TLC Sheets, Cellulose Brinkman code 5537, Brinkmann Instruments, Canada). Ascending chromatography was done and chromatographic separation of glucosamine and glucosamine 6 P was achieved by using a solvent consisting of 52 ml n-butanol, 32 ml of ethanol and 18 ml of distilled water. Glucosamine-6-P remained at the origin while glucosamine moved to some distance. The spots

were developed by spraying with a color reagent (made up of 0.6 g. p-anisidine and 0.8 g. pthalic acid in 5 ml of methanol) and then heating at 110° until color developed.

Cell Free Extracts

Samples were taken under various growth conditions, washed twice in cold distilled water and stored in pellet form at -20° for one week. The cell mass was then thawed in buffer (tris-Hcl, 0.05M, pH 7.4) and adjusted to a known volume before two successive passages through a prechilled French pressure cell at 23,000 pounds p.s.i. according to the method of Ball (1969). The resulting suspension was centrifuged to remove cell debris (2000 x g for 10 minutes) at 0°; the cloudy supernatant was aliquotted into small glass tubes and stored at -20° for subsequent enzyme assay.

Enzyme Assays

All assays were done on French press extracts using a temperature controlled Gilford 2000 recording spectrophotometer maintained at 30°.

Hexokinase (ATP glucose -6-phosphotransferase E.C. 2.7.1.2.) was measured according to Bergmeyer (1963). The reaction was coupled to NADP using glucose -6-phosphate dehydrogenase. The increase in absorbance at 340 nm due to NADPH was measured. $E = 6.22 \times 10^{-3} M^{-1} cms^{-1}$. Each cuvette contained 8.3 µg/ml glucose -6-phosphate dehydrogenase, 3.3 mM glucose, 8.3 mM Mg SO4. 8.3 mM ATP, 0.31 mM NADP and 0.23 M Tris-Hcl buffer (pH 7.4) in a total volume

of 1.5 ml. Under these conditions OD_{340}/min was proportional to the enzyme concentration.

Cytochrome c oxidase (Cytochrome c: Oxygen oxidoreductase, E.C.1.6.2.1.) was assayed by measuring the oxidation of cytochrome c at 550 nm, according to the method of L. Smith (1955). Each cuvette contained horse heart Type III cytochrome c (0.75 mg) in 1.5 ml of Tris-Hcl buffer (0.1M, pH 7.4). Cyrochrome c was reduced with ascorbic acid such that the ratio $O.D._{550}$:$O.D._{565}$ was between 12:1 and 14:1. Data are expressed as 1st order rate constant.

Samples were assayed three times for each sample and the values recorded in the results are the average for the three estimates. Specific activities of the enzymes were calculated by dividing the total activity per millilitre of extract by the protein content (mg/ml of extract).

Glucosamine Kinase The reaction mixture contained 4.0 mM glucosamine, 10mM $MgSO_4$, 20mM ATP and radioactive glucosamine (3 x 104 dpm/μmole) in a total volume of 1.0 ml. After addition of enzyme (0.2 ml) samples were taken at 30 second intervals and transferred to a tube containing 80% ethanol, 0.5 mg glucosamine and 0.5 mg glucosamine -6-P04. The ethanol extracts were chromatographed as described above to separate the reactant from the product. Under these conditions the rate of phosphorylation was linear for 120 seconds (Appendix Fig. 3).

Chemicals

Bacto agar, Bactopeptone, yeast extract and dextrose were purchased from Difco laboratories, Detroit, Michigan, U.S.A. ^{14}C-glucose, ^{14}C-glucosamine and ^{14}C-sorbose were obtained from the Radiochemical Centre, Amersham, U.K. ATP, Cytochrome C, NADP and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Company, St. Louis, U.S.A. All other reagents were of the highest grade available from BDH Ltd., Toronto, Canada.

CHAPTER 3

RESULTS

(1) Growth Characteristics

(i) Aerobic Growth on Non-fermentable Carbon Sources

Growth on 3% glycerol (YPG) is characterized in 4B2 by a 13 hour lag period followed by a 9 hour growth phase (Fig. 4). GR6 and $10P_3r$ show a 15 hour lag period followed by a 7 hour growth phase. The growth rates of these two mutants are similar to wild type. GR81 and GR108 show 16 and 18 hour lag periods and the growth rates of these two mutants are slower than wild type. The growth rates on glycerol medium are shown in Table I. All strains grow logarithmically on glycerol medium (Fig. 4).

(ii) Growth on Fermentable Carbon Sources

Growth on 3% glucose (YPD) is characterized by a 12 hour lag period followed by a fast 8 hour long logarithmic fermentative phase in 4B2 (Fig. 5). In the mutants GR6, $10P_3r$ and GR108 the lag phase is longer, 14, 15, and 16 hours respectively. The fermentative logarithmic growth rates of these mutants are similar to 4B2 and this growth phase is also 8 hours long. In mutant GR81 the lag phase is 18 hours long and the fermentative growth rate is slower. The fermentative logarithmic phase is 11 hours long.

LEGEND

Fig. 4: Aerobic Growth on Non-fermentable Carbon Source (YPG)

Yeast grown on YPD slopes for 24 hours at 30° were inoculated into YPG medium. Cultures were aerated by vigorous shaking at 350 rpm and the temperature was maintained at 30°.

3% Y P Glycerol

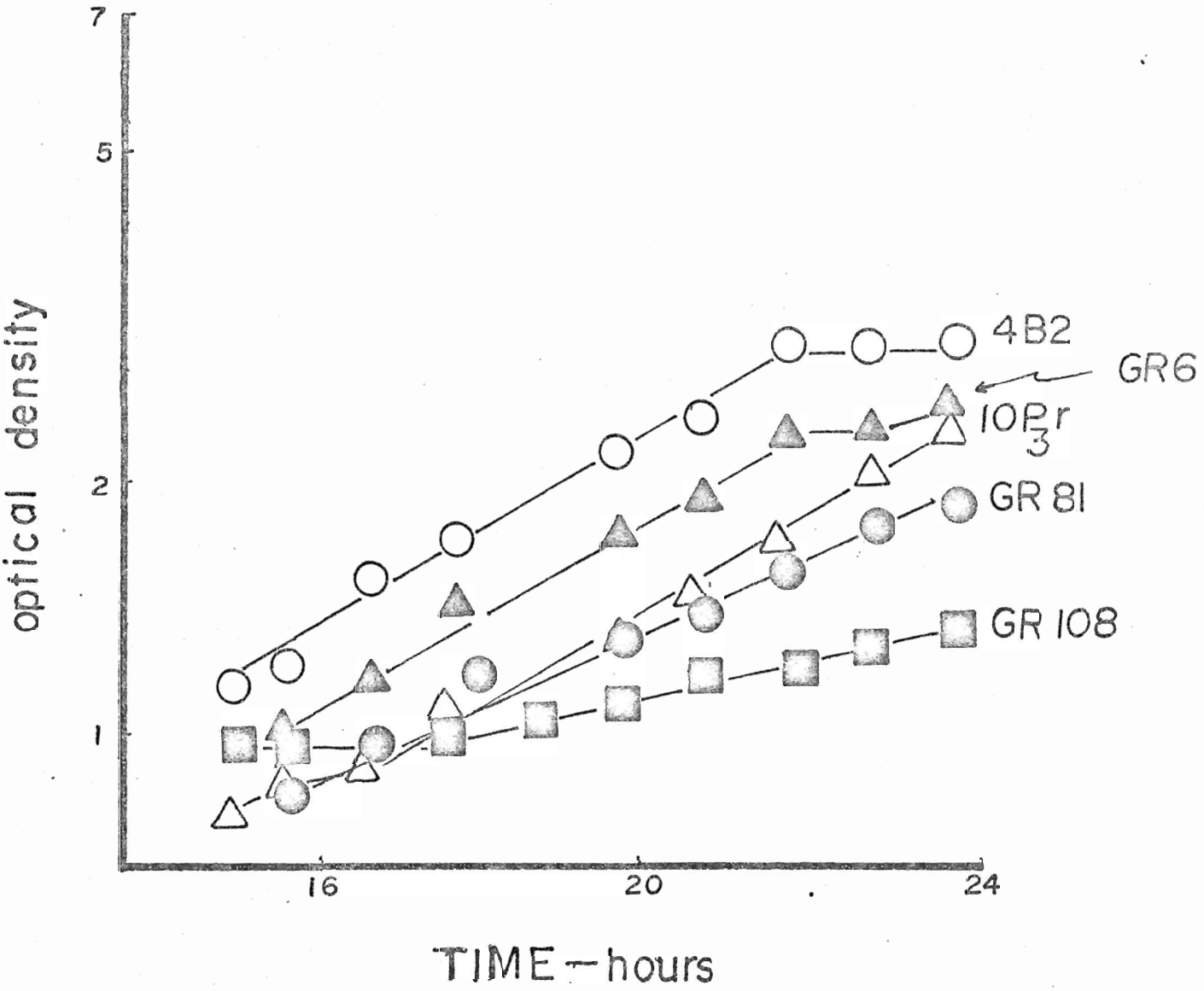


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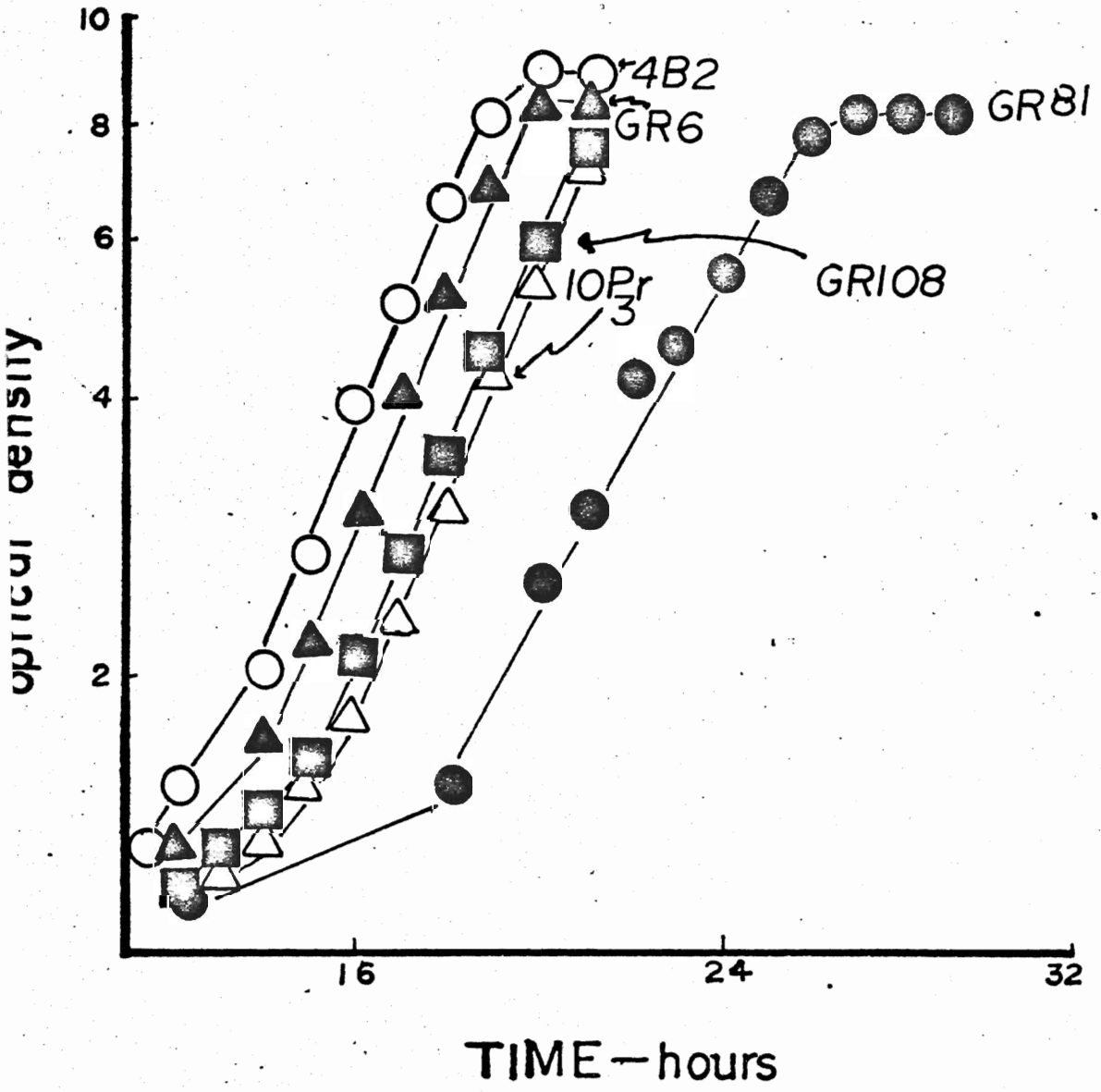
Fig. 5: Aerobic Growth on Fermentable Carbon Source (YPD).

Yeast grown on YPD slopes for 24 hours at 30° were inoculated into YPD medium. Cultures were aerated by vigorous shaking at 350 rpm and the temperature was maintained at 30°.

3% YPDextrose



The growth rates of 4B2 and its mutants are given in Table I. The yield (mg protein/ml) of the cultures is almost the same in 4B2 and the mutants when fermentative phase cells enter the slow growth phase (in slow growth phase ethanol is respired as the sole energy source). Data was not collected on this phase because we were interested in glucose repression (see below) and YPG growth rates had already been determined.

Growth of GR6 and $10P_3r$ was not tested on maltose and galactose because the growth of these mutants was similar to 4B2 on both glycerol and glucose media. Growth of 4B2 on 3% maltose (YP mal) is characterised by a 15 hour lag period and an 8 hour logarithmic fermentative phase (Fig. 6). GR108 shows an 18 hour lag period followed by an 8 hour fermentative phase. In GR81 the growth is characterised by a 24 hour lag period and 11 hours of fermentative growth. Both GR81 and GR108 grow logarithmically like 4B2. The growth rates are given in Table I. GR81 shows a slower growth rate on maltose which is similar to the rate on glucose. The pattern of growth for 4B2 and its mutants on maltose is similar to that for glucose and the yield/unit carbon source is similar.

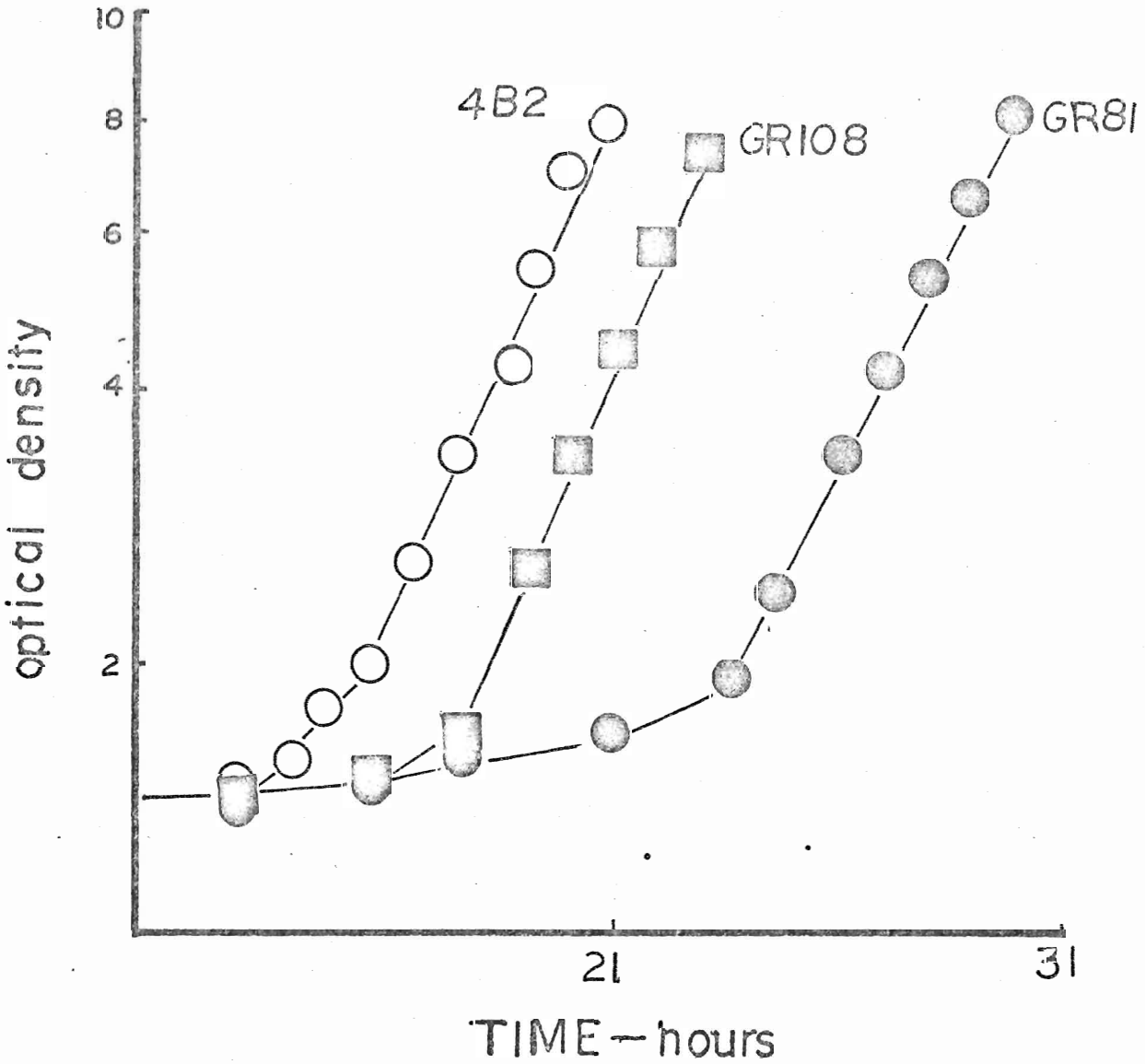
Growth of 4B2 on 3% galactose medium (YPgal) is characterised by a 13 hour lag period followed by a 10 hour long logarithmic fermentative phase. GR108 and GR81 show longer lag periods than 4B2, 15 and 19 hours

LEGEND

Fig. 6: Aerobic Growth on Fermentable Carbon Source (YPmal).

Yeast grown on YPD slopes for 24 hours at 30° were inoculated into YPmal medium. Cultures were aerated by vigorous shaking at 350 rpm and the temperature was maintained at 30°.

3% YPMaltose



LEGEND

Fig. 7: Aerobic Growth on Fermentable Carbon Source (YPgal).

Yeast grown on YPD slopes for 24 hours at 30° were inoculated into YPgal medium. Cultures were aerated by vigorous shaking at 350 rpm and the temperature was maintained at 30°.

3% YP Galactose

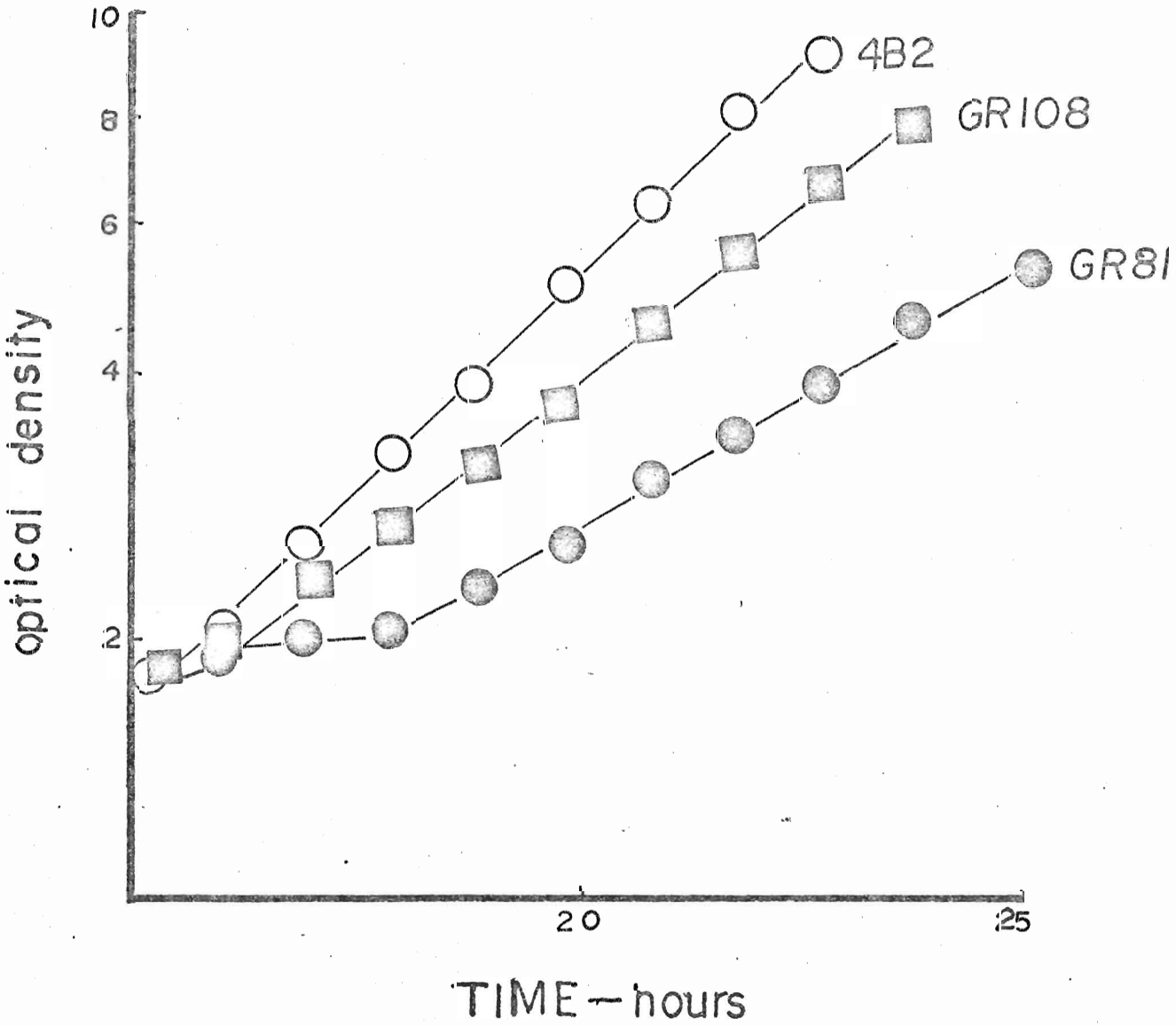


TABLE I: Growth Rates on Various Carbon Sources

Yeast grown on YPD slopes for 24 hours at 30° were inoculated into growth medium (YPD, YPG, YPmal or YPgal). Cultures were aerated by vigorous shaking and the temperature was maintained at 30°. The following data are from representative growth curves.

Growth Rates on Various Carbon Sources

Strains Used	Growth Rates ($O.D._{415}$/hour) YPG	YPD	YPgal	YPmal
4B2	0.3	1.3	1.0	1.25
GR6	0.3	1.3	NT*	NT*
$10P_3r$	0.3	1.3	NT*	NT*
GR81	0.25	0.9	0.5	0.8
GR108	0.13	1.3	0.65	1.20

*NT = Not Tested.

respectively (Fig. 7). The fast growth phase for GR81 is 15 hours and for GR108 is 12 hours long. Like 4B2 both strains grow logarithmically. The growth rates are summarized in Table I.

(2) Respiration

(i) Non-fermentable Carbon Source

When yeast cells are grown on a non-fermentable carbon source, e.g. glycerol, the respiratory ability (QO_2) remains the same throughout the growth period, i.e. growth is limited by respiration (Fig. 8). The QO_2's of the wild type and its mutants are given in Table II.

(ii) Fermentable Carbon Source

The QO_2 of yeast cells grown on a fermentable carbon source, e.g. glucose, varies with time. The respiratory ability at different growth phases is given in Table II.

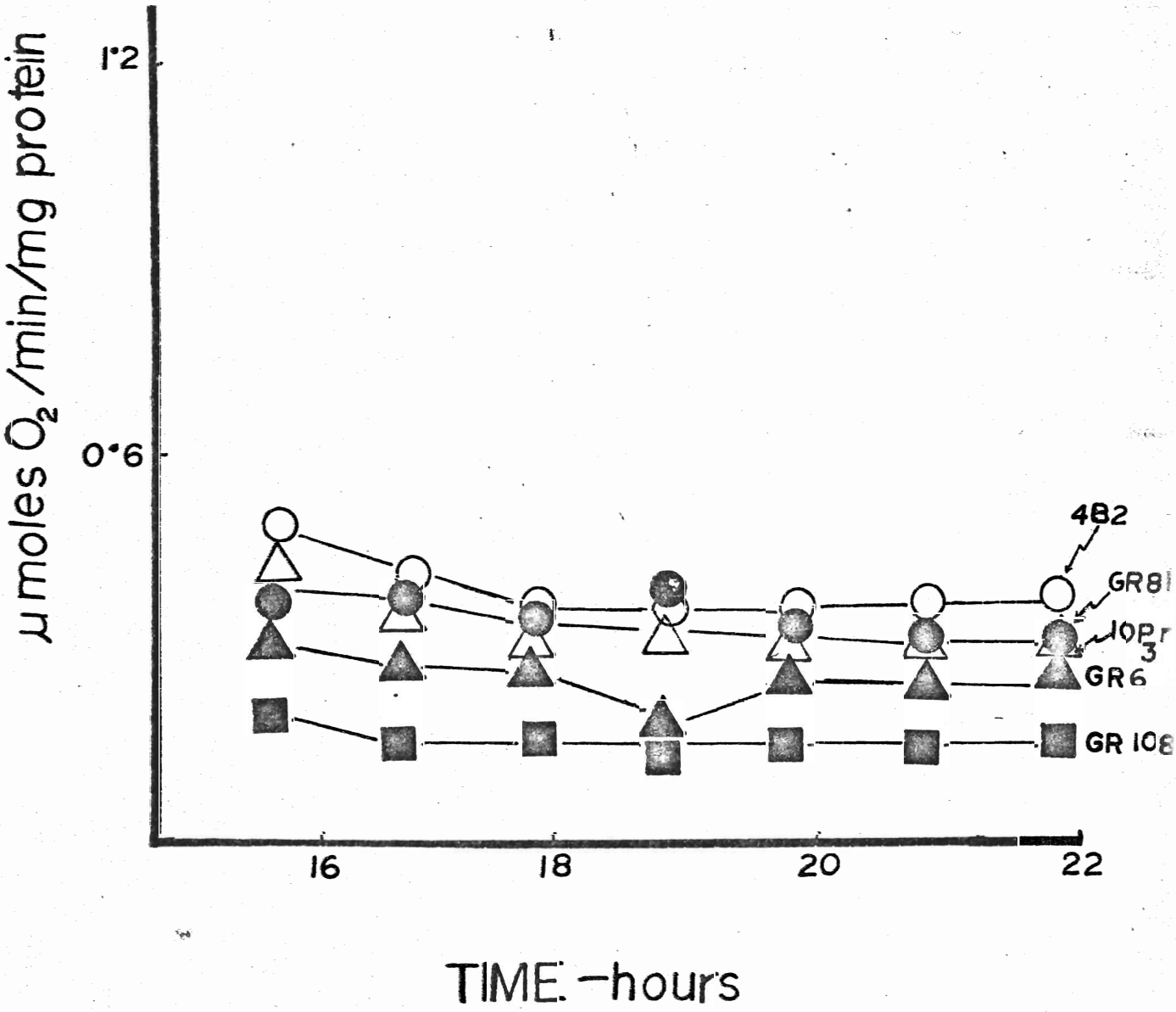
During early log phase the respiration is very low in all strains, because of repression of respiration by glucose. Derepression of respiration occurs 3 hours before the stationary phase in all strains except GR81 (Fig. 9). In the case of GR81 the respiration increases slowly throughout the growth phase and derepression of respiration occurs at stationary phase. During the derepression phase the rate of increase in respiration is very similar in 4B2, GR6, and $10P_3r$. GR108 shows a low final rate of respiration. At the beginning of stationary phase GR81 shows low

LEGEND

Fig. 8: Respiration (i) Non-fermentable Carbon Source.

The respiratory capability (QO_2) was monitored polarographically using a Clark-type electrode, on samples taken from aerobic yeast cultures. The culture conditions were YPG medium, 30° and aeration by vigorous shaking at 350 rpm.

3% Y P Glycerol



LEGEND

Fig. 9: Respiration (ii) Fermentable Carbon Source.

The respiratory capability (QO_2) was monitored polarographically on samples taken from aerobic yeast cultures. The culture conditions were YPD medium, 30^{o} and aeration by vigorous shaking at 350 rpm.

The arrows indicate time of glucose exhaustion.

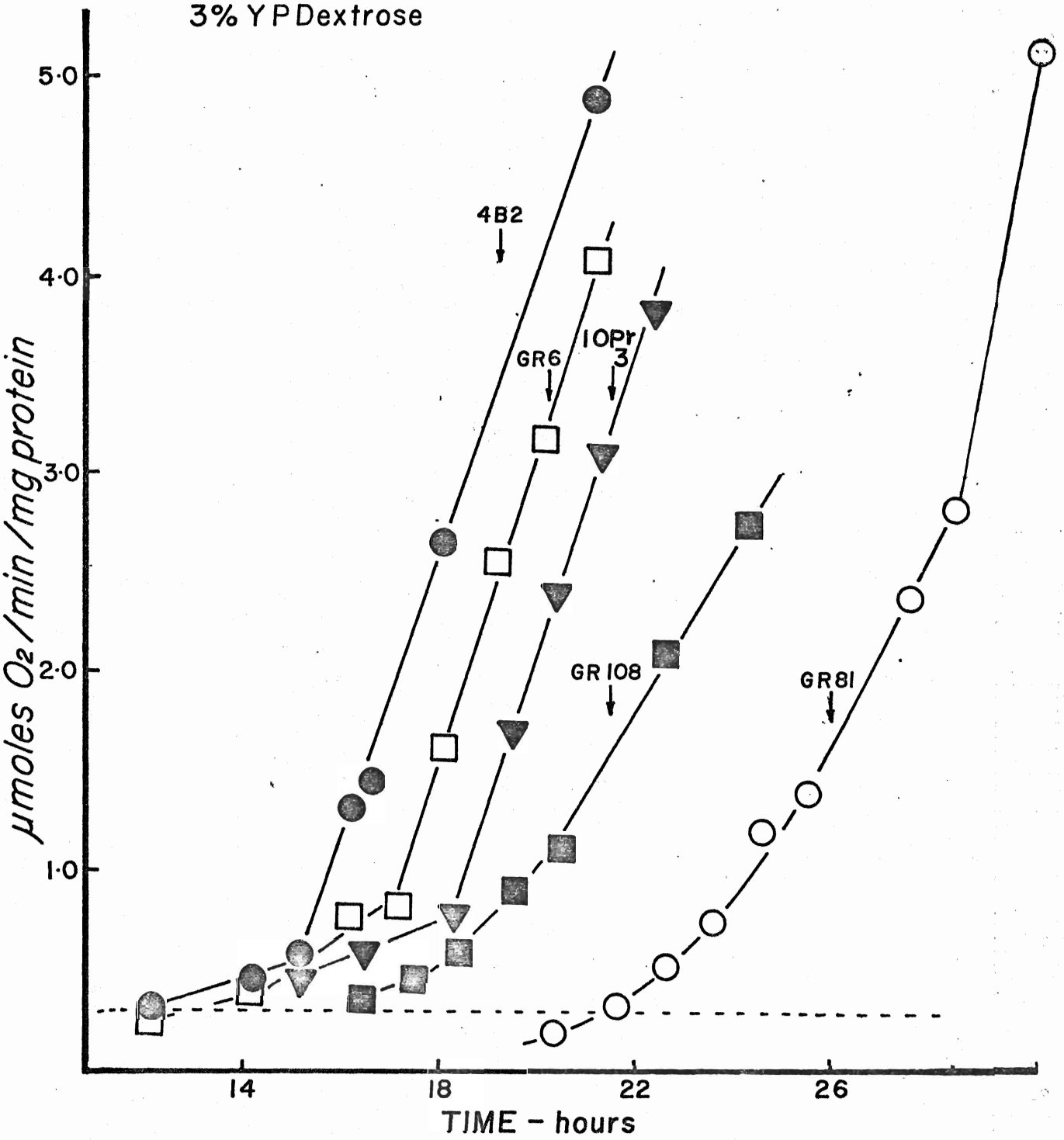

3% Y P Dextrose
5·0
4·0
3·0
2·0
1·0
μmoles O2 /min /mg protein
4B2
GR6
10Pr 3
GR 108
GR 81
14
18
22
26
TIME – hours

TABLE II: Respiratory Ability on YPG and YPD media

Yeast grown on YPD slopes for 24 hours at 30° were inoculated into growth medium (YPD or YPG). Cultures were aerated by shaking at 300 r.p.m. at 30°. Aliquots were taken at various intervals and oxygen consumption was measured polarographically.

Strains	Q_{O_2} (μmoles O_2/min/mg. protein)			
	YPG	YPD		
Used	All phases	Early log	Mid log	Plateau
4B2	0.45	0.35	1.30	2.70
GR6	0.30	0.30	0.90	2.50
$10P_3r$	0.35	0.50	1.50	2.75
GR10?	0.20	0.33	0.75	2.45
GR81	0.40	0.22	0.40	2.15

respiration but in a few hours it reaches the same level as 4B2.

The data presented in Figs. 4 - 9 and Tables I and II suggest that the fermentative and respiratory ability of mutants GR6 and $10P_3r$ are normal. This preliminary evidence also suggests that GR81 has some kind of lesion in glycolysis whereas GR108 has some kind of alteration associated with mitochondriogenesis.

(3) Sugar Transport

Glucosamine resistance in the mutants could easily be due to a lesion in the sugar transport mechanism, i.e., glucosamine does not enter the cells, so we looked at sugar transport next.

(i) Glucose Transport

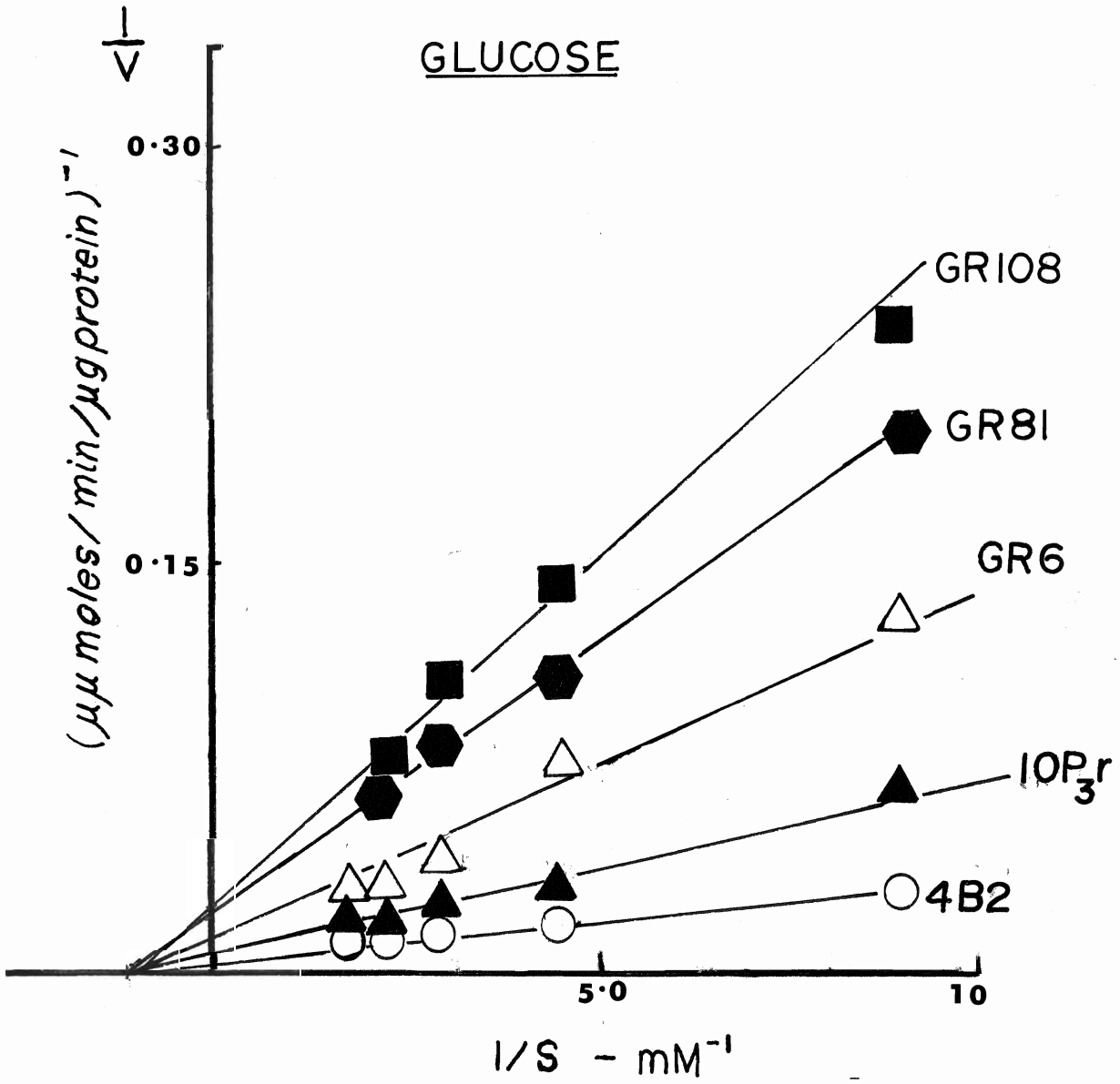
The glucose uptake was done in glycerol (YPG) grown, unwashed cells. The cells were taken at mid log phase and the uptake was observed for a time which gave linear uptake of glucose (Appendix Fig. 1).

The glucose uptake obeys Michaelis-Menton kinetics in 4B2 and all its mutants. The 1/S and 1/V graph (Line-Weaver - Burk plot) indicates that the Km remains the same in 4B2 and all mutants (Fig. 10). The Km for glucose is 0.75mM. The same Km means that carrier affinity for glucose is unaltered in all mutants. The Vmax is changed in all strains (Table III). Strain 4B2 shows the highest Vmax; Vmax 4B2 > GR6 > $10P_3r$ > GR81 > GR108.

LEGEND

Fig. 10: Glucose Transport.

Cells were grown to mid log phase on YPG and glucose transport was done on unwashed whole cells as described in the Methods.


GLUCOSE
1/V
0·30
0·15
(μμ moles/min/μg protein)⁻¹
GR108
GR81
GR6
10P3r
4B2
5·0
10
1/S - mM⁻¹

(ii) Glucosamine Transport

Glucosamine uptake was also done in glycerol grown mid-log phase cells. Cells were used directly for the transport experiment without washing. The uptake was observed for the time period which gave linear uptake for glucosamine (Appendix Fig. 2).

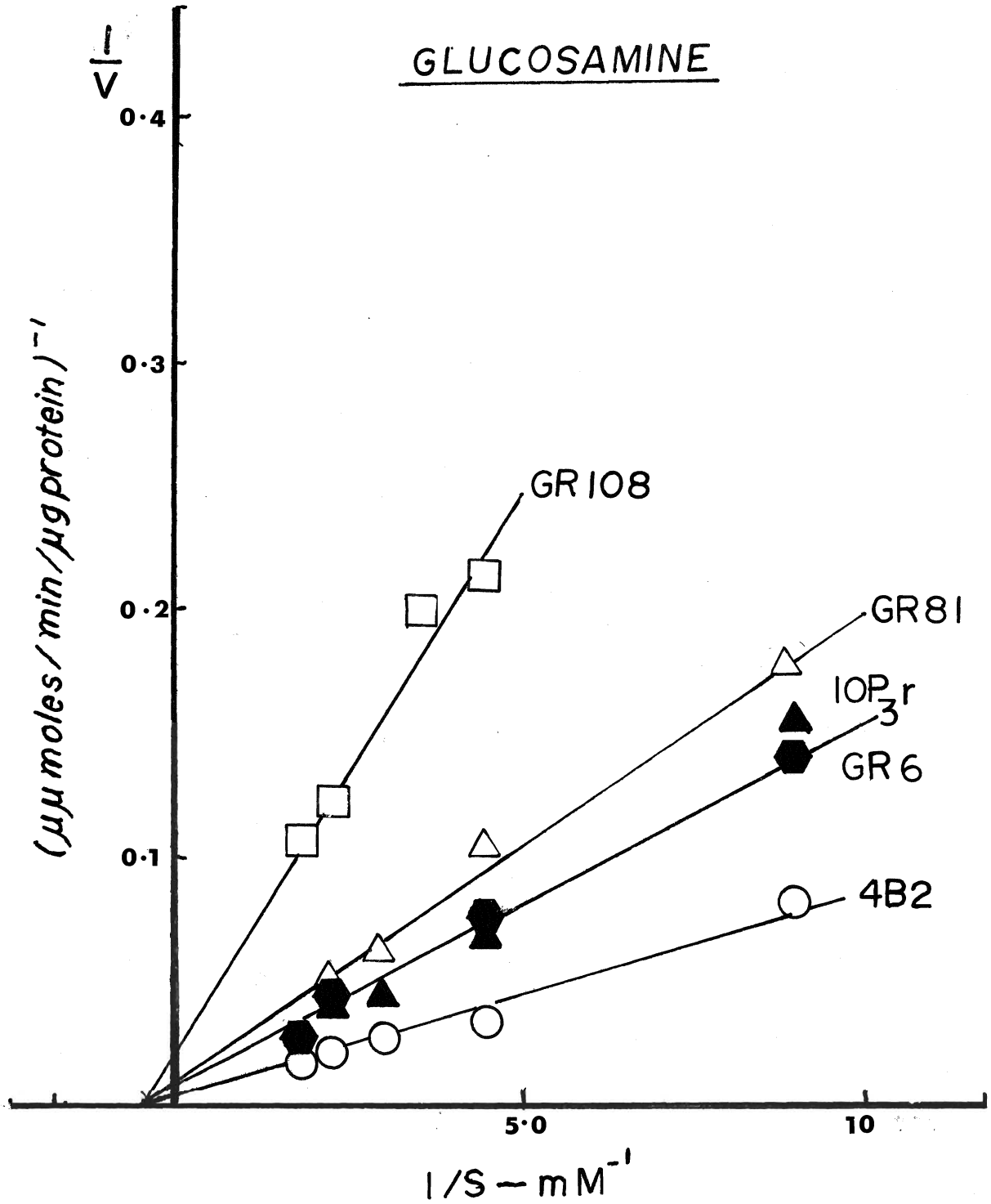
The glucosamine transport also shows Michaelis-Menton kinetics (Fig. 11). The Km for glucosamine is the same in 4B2 and all mutants, 2.2 mM, which is three times higher than Km glucose (Table III). Vmax (glucosamine) is changed in all strains (Table III). Vmax 4B2>GR6> $10P_3r$ > GR81 > GR108.

As glucose and glucosamine share the same carrier (Cirillo, 1961; Kotyk, 1967) it is not surprising that the affinity for carrier is unchanged in the mutants. The change in Vmax in the mutants could be due to a change in carrier or due to a change in energy metabolism as both glucose and glucosamine are phosphorylated using metabolic energy. Sorbose is also transported in yeast cells by fascilitated diffusion using the same carrier as glucose and glucosamine (Cirillo, 1962; VanSteveninck, 1966) but is not phosphorylated. So the sorbose uptake experiments were done in 4B2 and all mutants to distinguish between change in the carrier and change in energy metabolism in the mutants.

LEGEND

Fig. 11: Glucosamine Transport.

Cells were grown to mid log phase on YPG and glucosamine transport was done on unwashed cells as specified in Methods.


GLUCOSAMINE
1/V
0·4
0·3
0·2
0·1
(μμ moles/min/μg protein)⁻¹
GR108
GR81
IOP3r
GR6
4B2
5·0
10
1/S — mM⁻¹

LEGEND

Fig. 12: Sorbose Transport.

Cells were grown to mid log phase on YPG and sorbose transport was done on unwashed cells as specified in Methods.

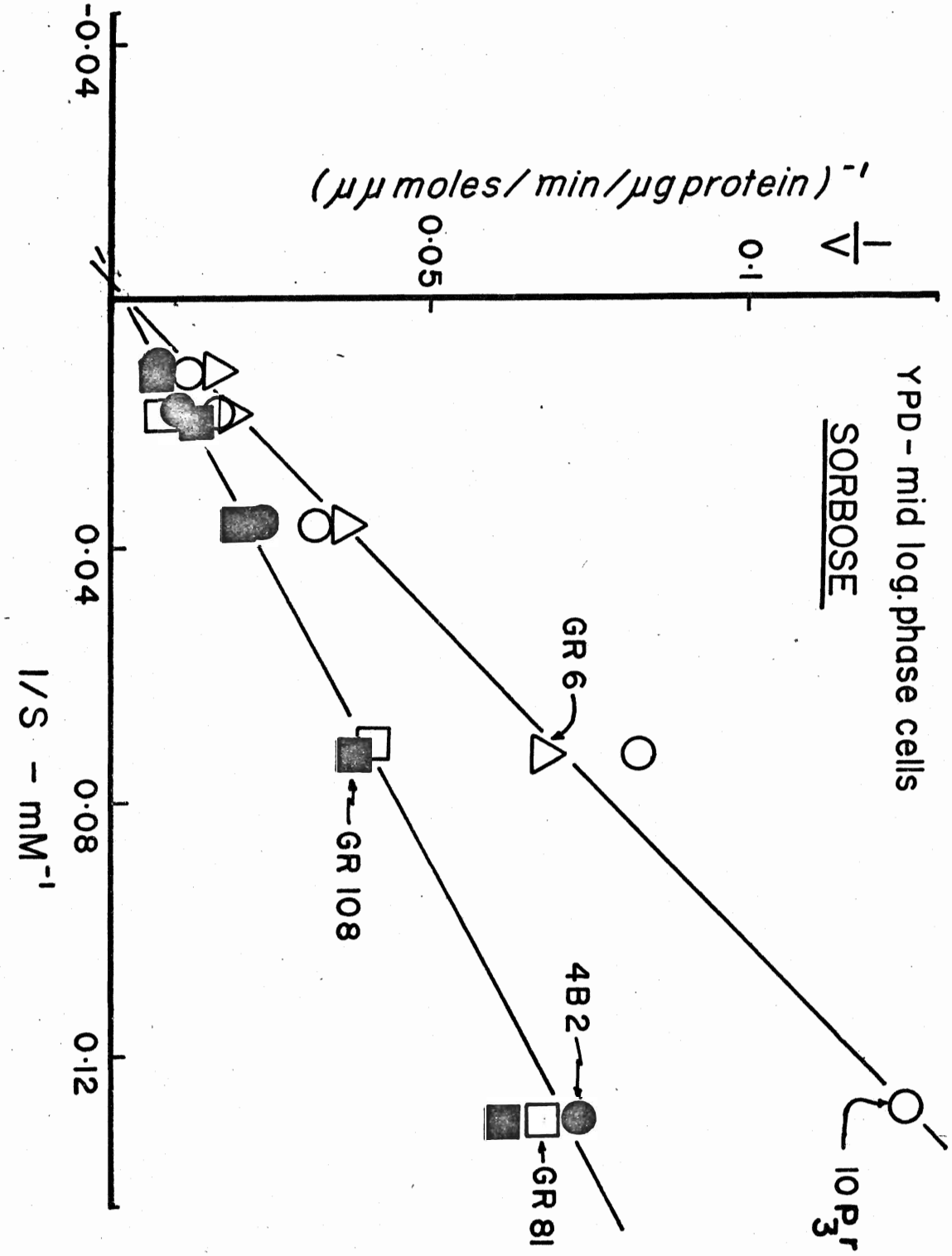

YPD - mid log. phase cells
SORBOSE
$\frac{1}{V}$ ($\mu\mu$ moles / min / μg protein)$^{-1}$
0·1
0·05
-0·04
0·04
0·08
0·12
1/S - mM^{-1}
GR 6
GR 108
4B2
GR 81
10 P$_3^r$

TABLE III: Transport Characteristics for Various Sugars

Yeast cells were grown in YPG medium and then glucose, glucosamine sorbose transport was done on unwashed cells as specified in methods.

Transport Characteristics for Various Sugars

Strains Used	Sorbose Km*	Sorbose Vmax**	Glucose Km	Glucose Vmax	Glucosamine Km	Glucosamine Vmax
4B2	250	500	0.75	200	2.2	400
GR6	250	250	0.75	135	2.2	200
$10P_3r$	250	250	0.75	90	2.2	150
GR81	250	500	0.75	44	2.2	200
GR10?	250	500	0.75	25	2.2	50

*Km is in mM.

**Vmax = picomoles/min/mg protein.

(iii) Sorbose Transport

Sorbose transport also follows Michaelis-Menton kinetics (Fig. 12). The uptake was not observed within five minutes, so cells were incubated in the presence of sorbose for five minutes and then linear uptake was observed. The Km (sorbose) is very high, that is 250 mM (Fig. 12) and it is the same for 4B2 and its mutants. The Vmax of 4B2, GR81, and GR108 is the same, 500 µµmoles/min./mg. protein. Both GR6 and $10P_3r$ show a lower Vmax (sorbose) which is 250 µµmoles/min./mg protein (Table III).

The data presented in Figs. 10, 11, and 12 and Table III suggest that GR6 and $10P_3r$ are probably transport mutants and the lower Vmax of GR81 and GR108 for phosphorylated sugars is probably due to change(s) in energy metabolism, i.e., a low phosphorylation rate.

(4) The Role of Phosphate and Phosphorylation

The following experiments were done to clarify the relationship between inorganic phosphate (Pi), phosphorylation and glucosamine poisoning in 4B2 and its mutants.

(i) Effect of Inorganic Phosphate

When inorganic phosphate was added to glycerol growing cells (YPG) an increase in oxygen uptake was observed (Figs. 13a, b, c, d, and e). In one hour 4B2 showed a 49% increase in oxygen uptake (Table IV). The mutants GR6, $10P_3r$, GR81, and GR108 showed 70, 80, 40 and

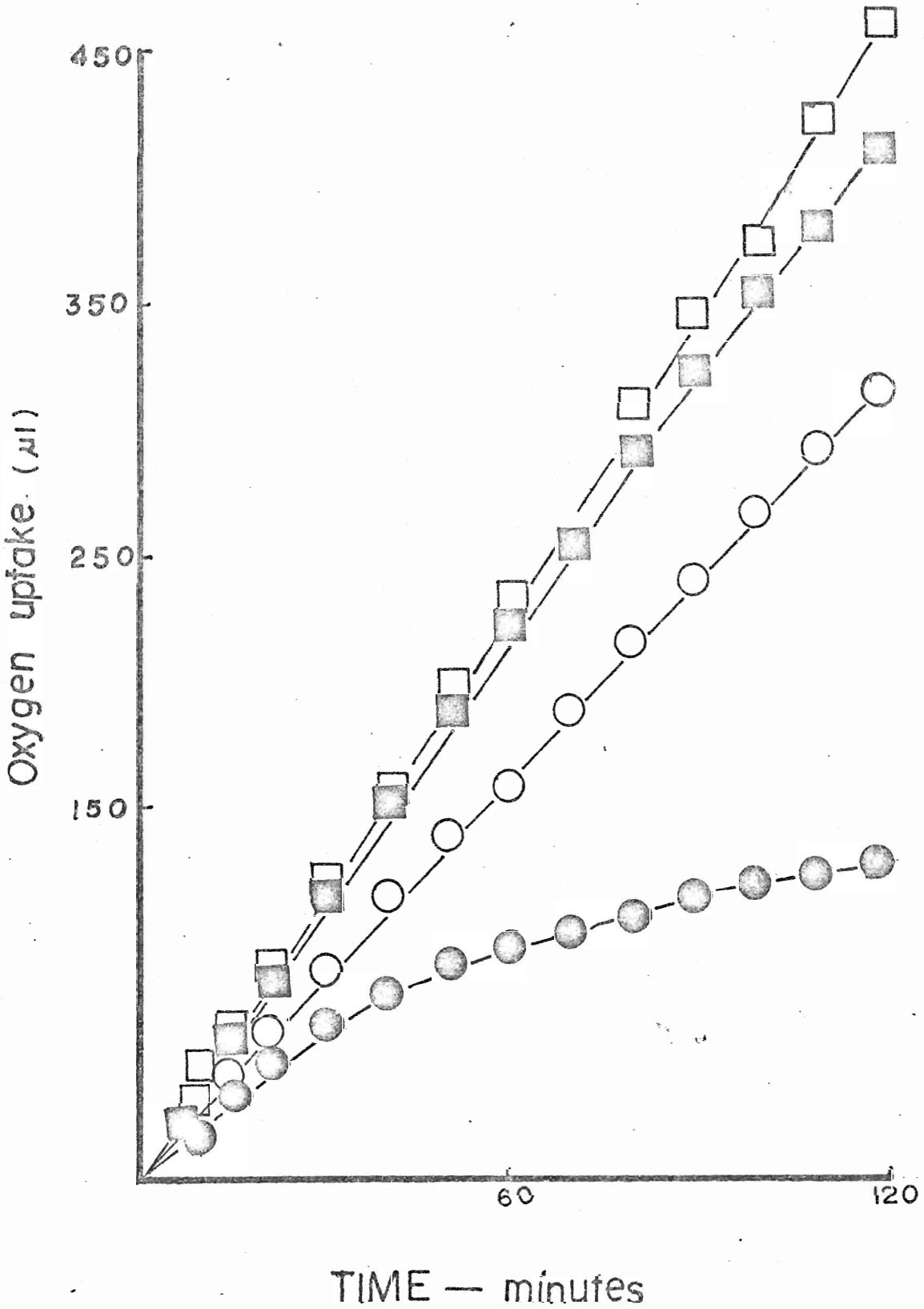
LEGEND

Fig. 13(a): Effect of Inorganic Phosphate (Pi) and Glucosamine.

Yeast were grown to mid log phase on YPG, harvested, diluted to $O.D._{415}$= 0.20 in fresh YPG and then used in the Gilson respirometer for oxygen uptake (see Methods).

- ○ Control
- ● Glucosamine (final concentration 0.05%)
- □ Pi (final concentration 0.066%)
- ■ Glucosamine + Pi

4B2



LEGEND

Fig. 13(b): Effect of Inorganic Phosphate (Pi) and Glucosamine.

Yeast were grown to mid log phase on YPG, harvested, diluted to $O.D._{415}$= 0.20 in fresh YPG and then used in the Gilson respirometer for oxygen uptake (see Methods).

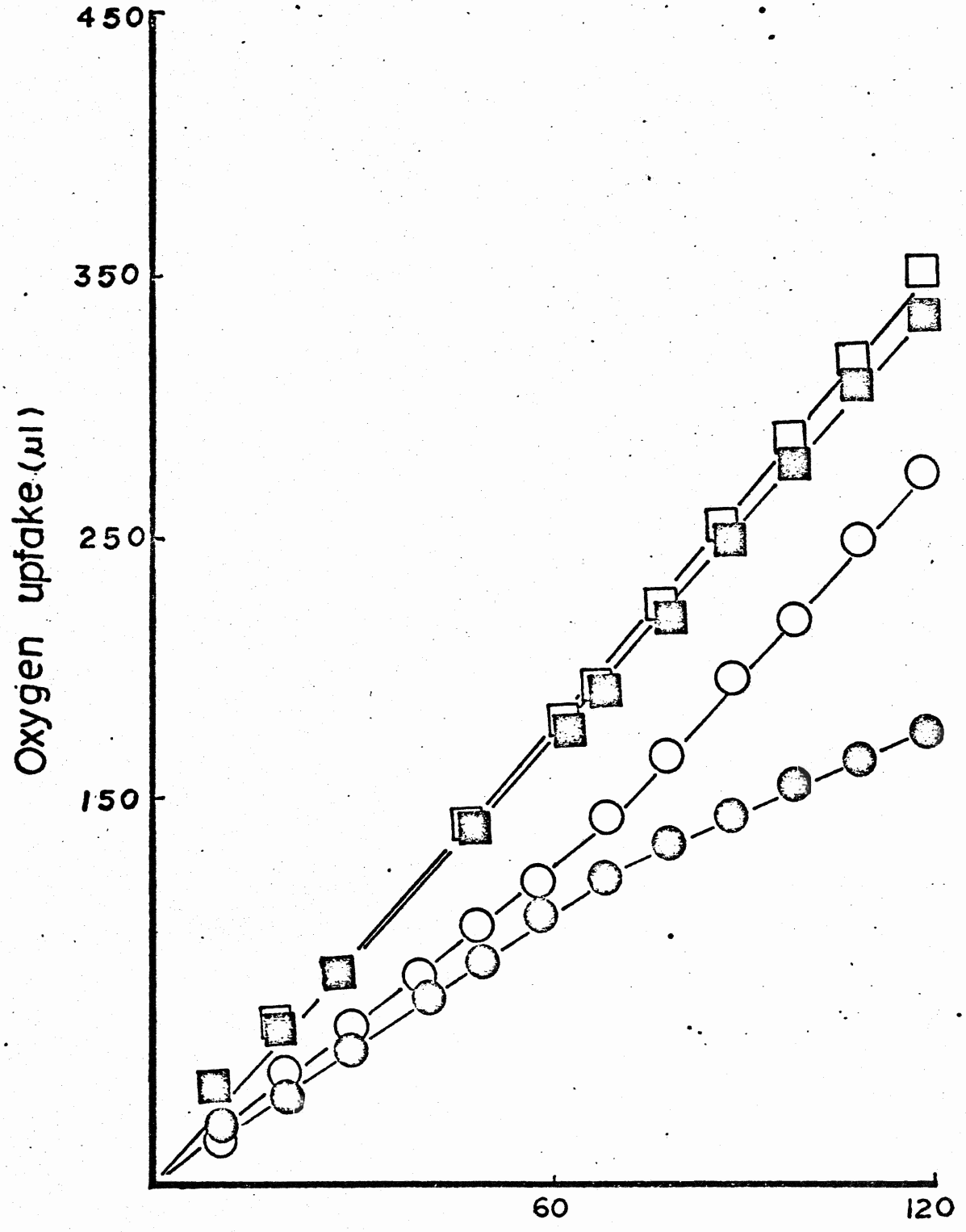
○ Control

● Glucosamine (final concentration 0.05%)

□ Pi (final concentration 0.066%)

■ Glucosamine + Pi

GR 81



LEGEND

Fig. 13(c): Effect of Inorganic Phosphate (Pi) and Glucosamine.

Yeast were grown to mid log phase on YPG, harvested, diluted to $O.D._{415}$= 0.20 in fresh YPG and then used in the Gilson respirometer for oxygen uptake (see Methods).

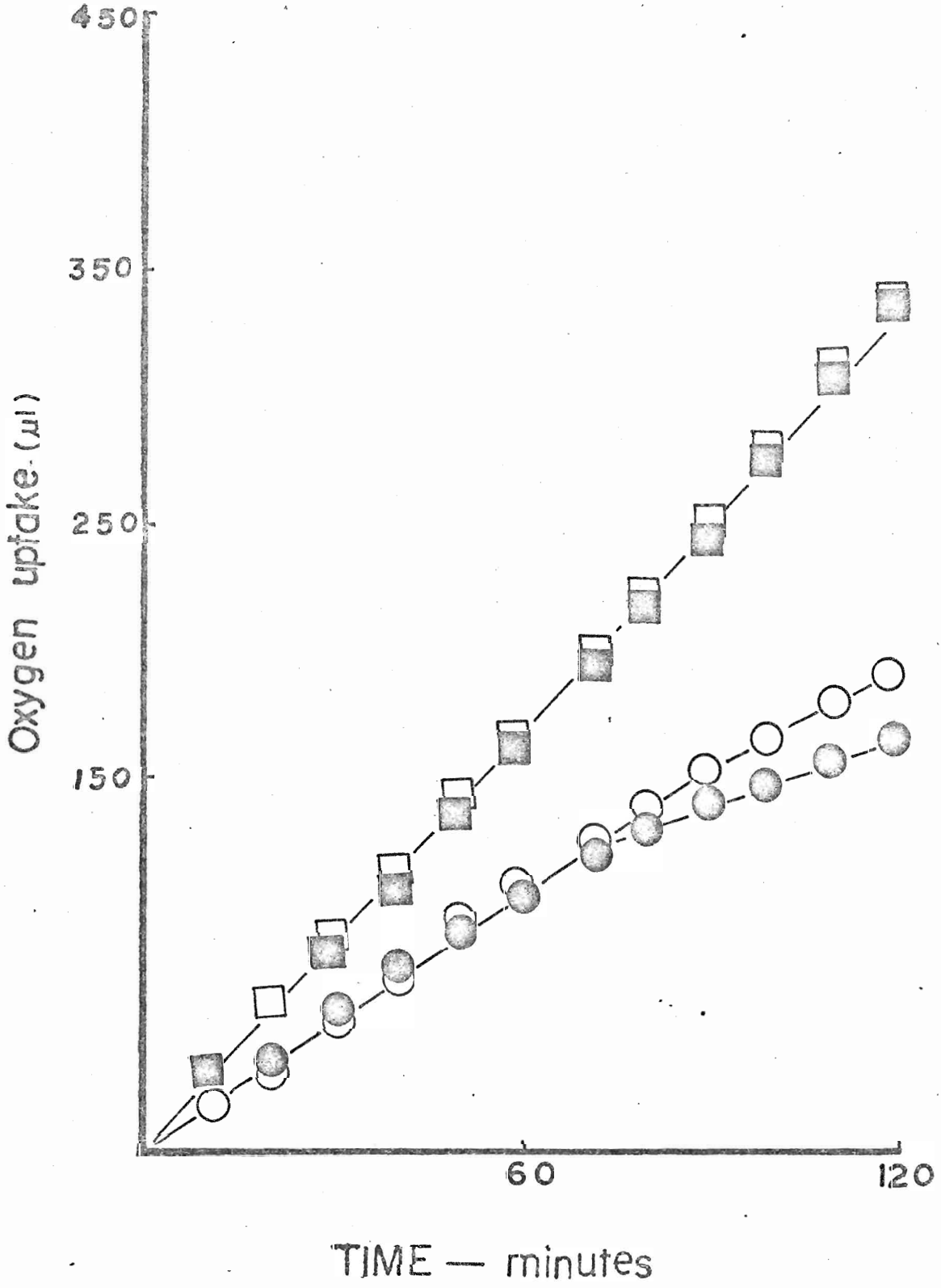
○ Control

● Glucosamine (final concentration 0.05%)

□ Pi (final concentration 0.066%)

■ Glucosamine + Pi

GR 108



LEGEND

Fig. 13(d): Effect of Inorganic Phosphate (Pi) and Glucosamine.

Yeast were grown to mid log phase on YPG, harvested, diluted to $O.D._{415}$= 0.20 in fresh YPG and then used in the Gilson respirometer for oxygen uptake (see Methods).

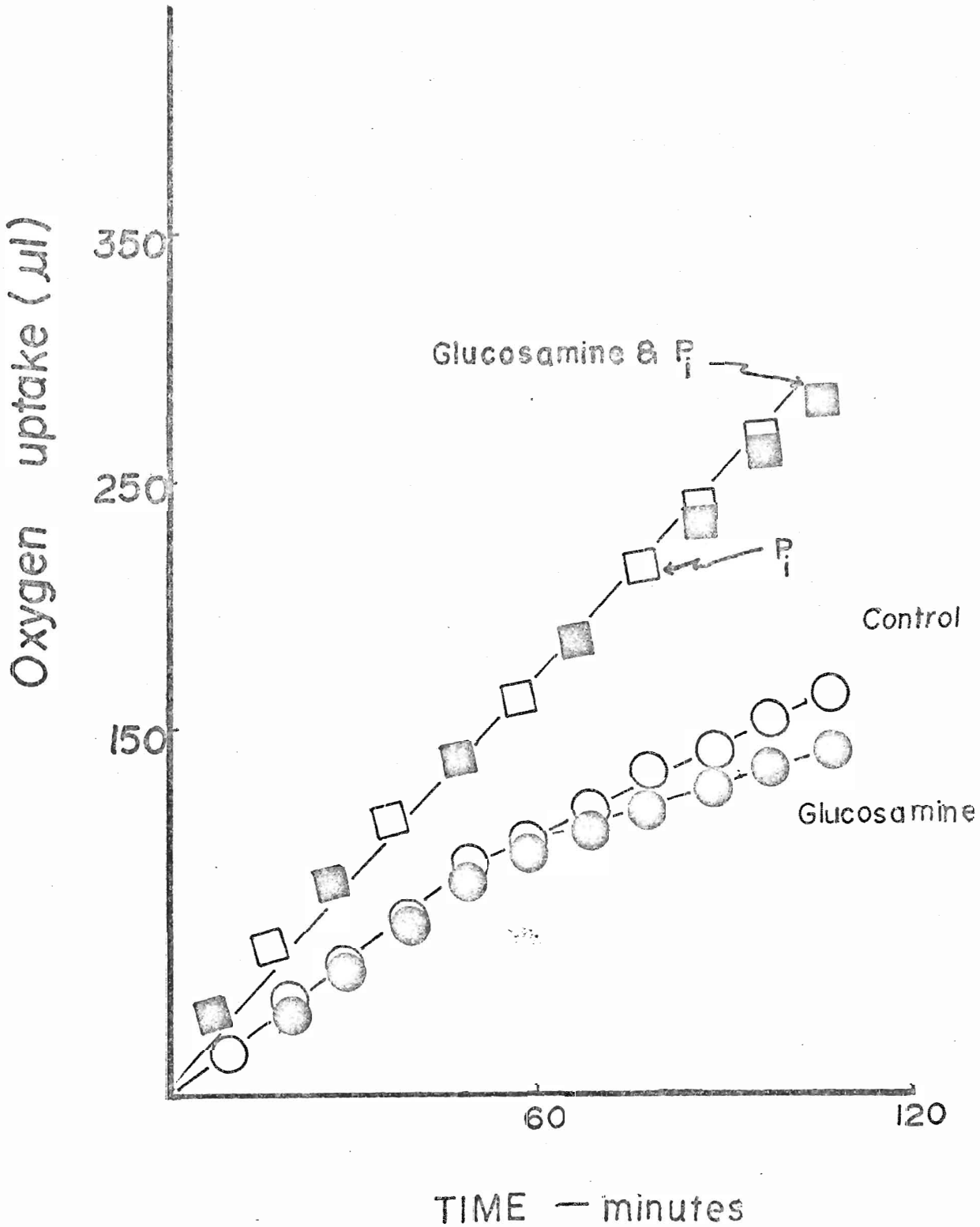
○ Control

● Glucosamine (final concentration 0.05%)

□ Pi (final concentration 0.066%)

■ Glucosamine + Pi

GR6


Oxygen uptake (µl)
350
250
150
Glucosamine & P_i
P_i
Control
Glucosamine
60
120
TIME — minutes

LEGEND

Fig. 13(e): Effect of Inorganic Phosphate (Pi) and Glucosamine.

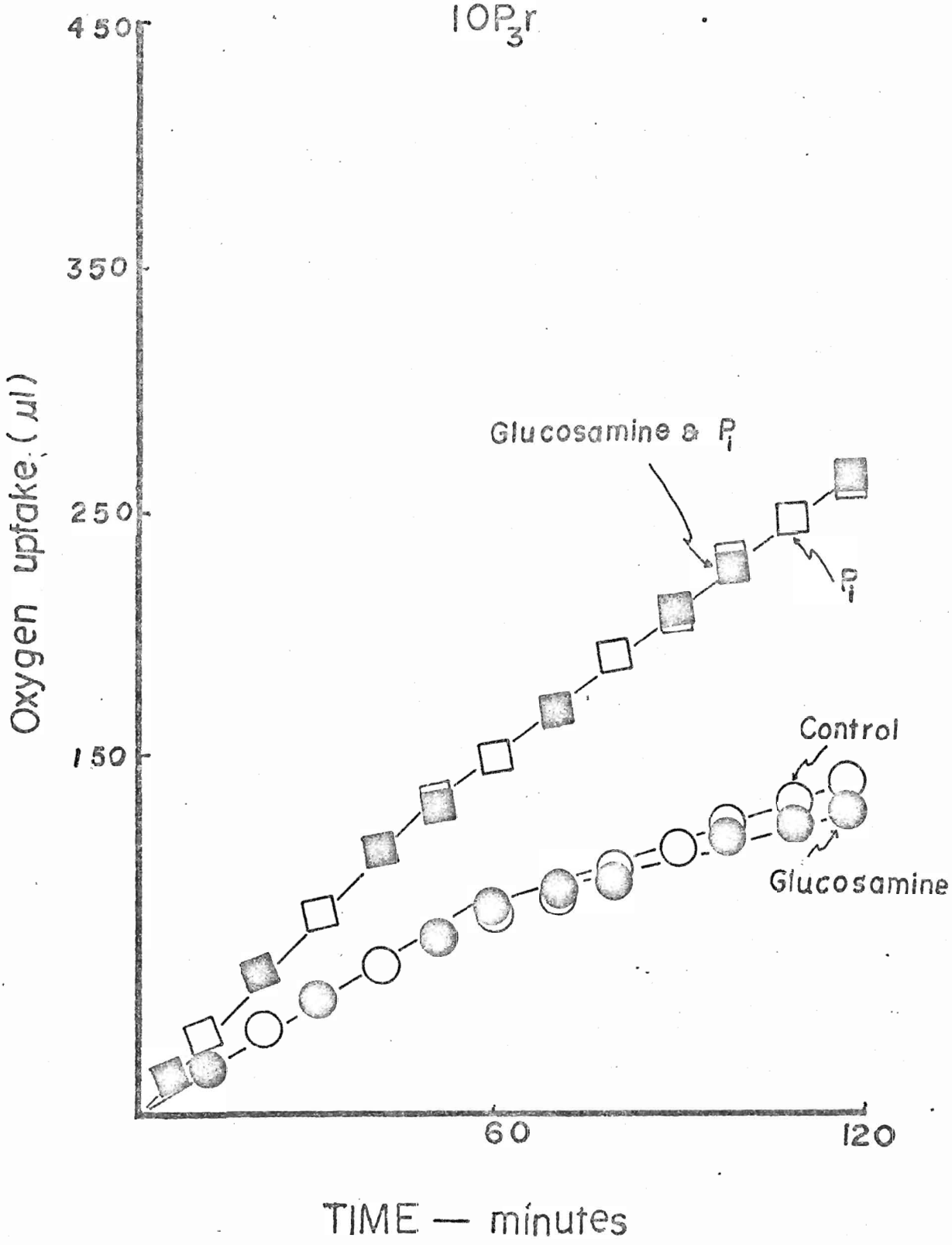
Yeast were grown to mid log phase on YPG, harvested, diluted to $O.D._{415}$ = 0.20 in fresh YPG and then used in the Gilson respirometer for oxygen uptake (see Methods).

○ Control

● Glucosamine (final concentration 0.05%)

□ Pi (final concentration 0.066%)

■ Glucosamine + Pi


10P3r
450
350
250
150
Oxygen uptake (µl)
Glucosamine & Pi
Pi
Control
Glucosamine
60
120
TIME — minutes

62% increase in oxygen uptake respectively (Table IV).

When 0.05% glucosamine was added to glycerol grown cells there was generally a decrease in oxygen uptake. In one hour there was no effect of glucosamine on $10P_3r$ and GR108 but 4B2, GR6, and GR81 showed 45, 6, and 20% inhibition respectively (Figs. 13 (a) to 13 (e) and Table IV). Sensitivity to glucosamine increased with time; by 2 hours 4B2, GR6, $10P_3r$, GR81 and GR108 showed 60, 15, 10, 36, and 20% inhibition respectively (Figs. 13 (a) to 13 (e)).

When Pi was added together with glucosamine to glycerol grown 4B2 cells, the cells were protected from glucosamine poisoning (Fig. 13 (a) and Table IV). At times greater than one hour a very slight drop in oxygen uptake was observed in the presence of glucosamine which suggests that phosphate can be exhausted by glucosamine metabolism. GR81 and the other mutants which are partially sensitive to glucosamine were also protected from glucosamine poisoning in the presence of Pi (Figs. 13 (b) to 13 (e) and Table IV).

All the above experiments indicate that YPG cells are Pi limited and that inorganic phosphate is essential for protecting wild type cells from glucosamine poisoning and that inhibition is caused by glucosamine-6-phosphate acting as a Pi sink.

TABLE IV: Effect of Glucosamine and Inorganic Phosphate on Respiration

Yeast were grown to mid log phase on YPG, harvested, diluted to O.D.415= 0.20 in fresh YPG and then used in the Gilson respirometer for O_2 uptake (see methods). Additions were made as indicated.

Effect of Glucosamine and Inorganic Phosphate on Respiration

Strains Used	Oxygen Uptake (% of control)			
	Control	$GLNH_2$	Pi	$GLNH_2$+Pi
4B2	100*	55	149	140
GR6	100	94	170	165
$10P_3r$	100	100	180	180
GR81	100	80	140	140
GR108	100	100	162	162

*Calculated after 60 minutes

(5) Hexokinase

The effect of inorganic phosphate and the transport experiments indicate that 4B2 is poisoned by glucosamine due to a greater utilization of ATP (and Pi). This greater utilization of ATP could be due to a higher specific activity of hexokinase in 4B2 than in the mutants. The following experiments were done to see whether hexokinase is the same in all mutants or not.

(i) Hexokinase (glucokinase) activity was measured spectrophotometrically on French press extracts of glucose and glycerol grown mid log phase cells. Glucose grown cells showed more hexokinase activity than glycerol grown cells (Table V). Hexokinase activity was the same in either glucose or glycerol grown cells (Table V).

The Km and Vmax for glucose and ATP were also determined which were the same in all three strains tested (Fig. 14a and 14b).

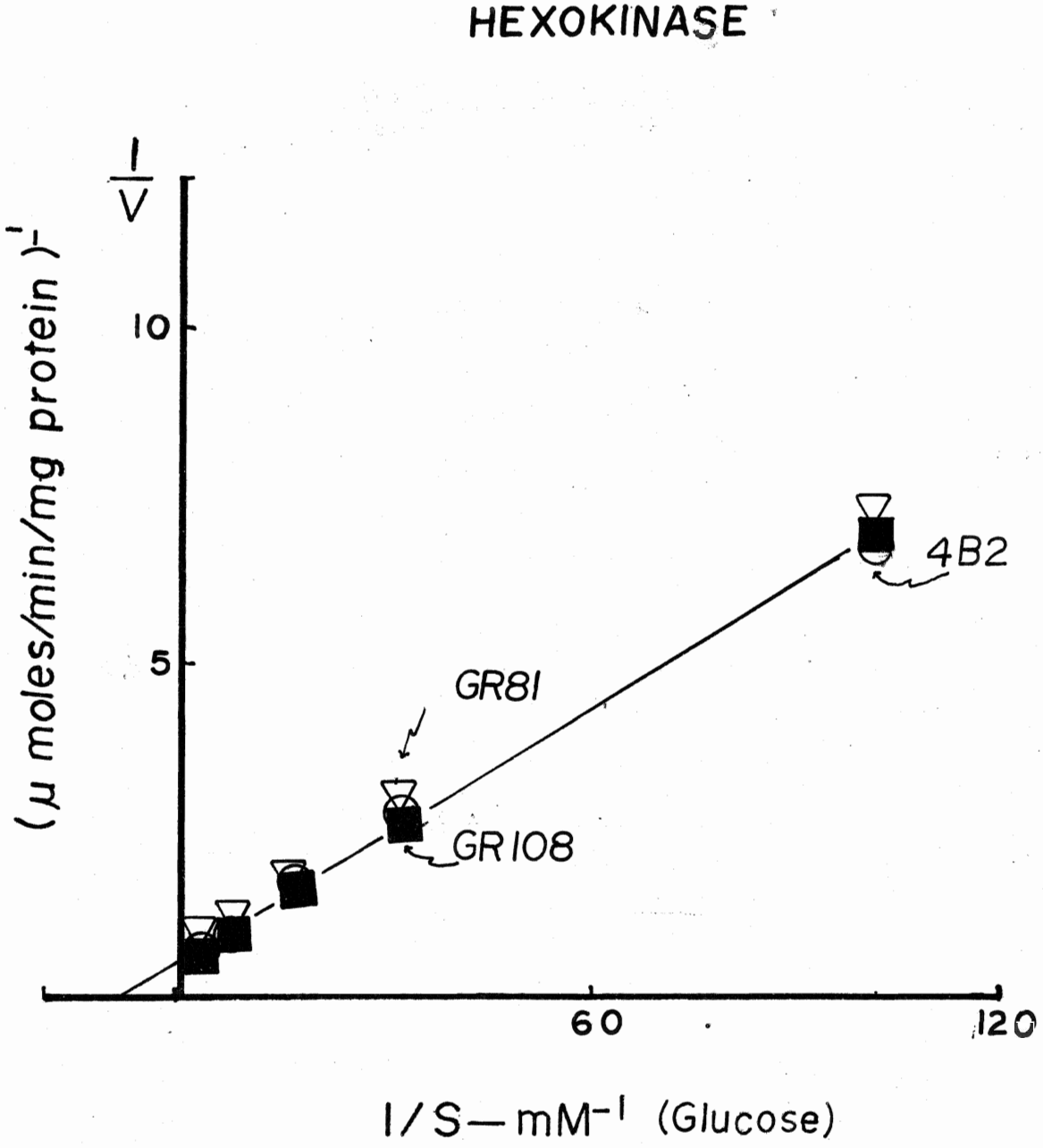
(ii) Glucosamine kinase activity was determined *in vivo* and *in vitro* using a ^{14}C assay of glucosamine kinase activity. The *in vitro* phosphorylation was done on French press extracts. The glucosamine kinase activity was similar in all strains tested (Table VI).

The *in vivo* phosphorylation study showed a difference

LEGEND

Fig. 14(a): Hexokinase.

Km and V_{max} for glucose for hexokinase were determined on French-pressed cell free extracts of cell grown to mid log phase in YPD.


HEXOKINASE
1/V
(μ moles/min/mg protein)⁻¹
10
5
4B2
GR81
GR108
60
120
1/S — mM⁻¹ (Glucose)

LEGEND

Fig. 14(b): Hexokinase.

Km and V_{max} for ATP, for hexokinase were determined on French-pressed cell free extracts of cell grown to mid log phase in YPD.

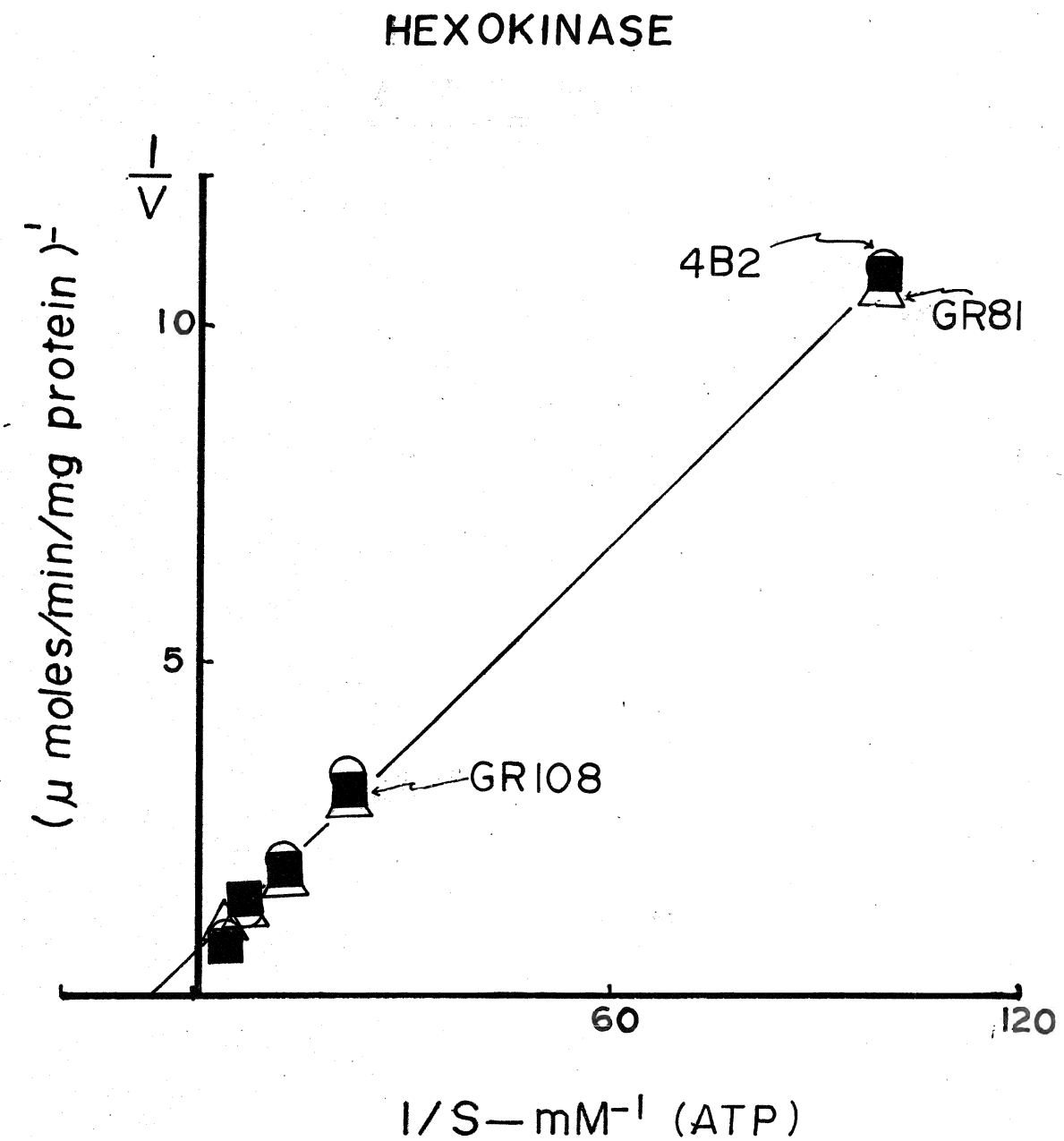

HEXOKINASE
1/V
(μ moles/min/mg protein)⁻¹
10
5
4B2
GR81
GR108
60
120
1/S—mM⁻¹ (ATP)

TABLE V : Hexokinase Activity

Activities were determined on French-pressed cell free extracts of cells grown to mid log phase in YPD (repressed) and YPG (derepressed) media.

Strains Used	Hexokinase Activity (µmoles/mg protein) Repressed	De-repressed
4B2	0.24	0.13
GR81	0.24	0.18
GR108	0.23	0.16

TABLE VI : In Vitro Phosphorylation or Glucosamine Kinase

Activities were determined on French-pressed cell free extracts of cells grown to mid log phase in YPG. Activity was determined by using ^{14}C assay as specified in the methods.

In Vitro Phosphorylation or Glucosamine Kinase

Strains Used	Activity (µmoles/mg protein)
4B2	0.060
GR81	0.067
GR108	0.059

TABLE VII : In Vivo Phosphorylation

Yeast cells were grown in YPG medium, and then glucosamine transport was done on unwashed cells. Radioactive samples from transport experiments were chromatographed. Glucosamine and glucosamine-6-phosphate were determined as specified in methods.

In Vivo Phosphorylation

Strain Used	Time (Sec.)	Total (cpm)	Origin	%$GLNH_2$-6-PO_4
4B2	100	259	132	51
	300	617	327	53
	500	918	358	39
GR81	100	260	79	30
	300	576	207	36
	500	889	264	29
GR108	100	112	35	31
	300	154	48	32
	500	254	70	29

in phosphorylation of glucosamine in all strains (Table VII). The initial rate of phosphorylation of glucosamine in 4B2 was about 1.5 times higher than GR81 and GR108 (Table VIII).

(6) Cytochrome Oxidase

Preliminary experiments indicate that GR108 has a slow growth rate on YPG and its respiratory ability is always low in both YPG and YPD. This could be due to less mitochondrial enzymes and finally less ATP production on YPG. Cytochrome oxidase, which is one of the main enzymes of the mitochondrion might give some indication that GR108 has some kind of lesion in mitochondriogenesis.

Cytochrome oxidase was measured in French press extracts of glucose grown (repressed) and YPG (derepressed) cells. The derepressed cells show higher activity than repressed cells. The cytochrome oxidase activity of 4B2, GR6, $10P_3r$ and GR81 were very similar. GR108 showed a lower activity in both the repressed and derepressed conditions than wild type (Table VIII).

TABLE VIII: Cytochrome Oxidase

Activities were determined on French-pressed cell free extracts of cells grown to mid log phase in YPD (repressed) and YPG (derepressed) media.

Cytochrome Oxidase

Strains Used	Activity (μmoles/mg protein)	
	Repressed	Derepressed
4B2	0.63	1.9
GR6	0.59	1.59
$10P_3r$	0.54	2.10
GR81	0.57	1.3
GR108	0.38	0.66

CHAPTER 4

DISCUSSION

As discussed in the earlier sections on catabolite repression and the Crabtree effect (see literature review) the mechanisms leading to respiratory repression in yeast are not yet clearly understood. It is also not clear as to whether or not there is some or no connection between repression of mitochondriogenesis and the Crabtree effect.

Because glucosamine also causes both effects, by using glucosamine resistant mutants an attempt can be made to find out the mechanisms which cause these effects. Due to the limited metabolism of glucosamine by yeast cells (see introduction) only four major biochemical functions exist, mutation of which could alter cellular metabolism to confer glucosamine resistance.

(i) Transport of glucosamine into the cells

(a) facilitated diffusion (Cirillo, 1962 and 1970; Kotyk, 1973) or

(b) active transport (Van Steveninck, 1968)

(ii) Hexokinase, which phosphorylates the sugar inside the cells using ATP (Cirillo, 1970; Kotyk, 1973)

(iii) ADP - ATP translocating system in the mitochondrion.

(iv) Enzymes involved in the interconversion of adenine nucleotides.

Reactions (i) and (ii) are present in the cytosolic component while the other two are present in the mitochondrion. So it is very unlikely that mitochondrial mutants will cause mutation in (i) and (ii) since this involves modification in cytosolic components. The mutants I used for study are nuclear and cytoplasmic mutants so mutation of any of the above reactions is possible.

As it is commonly agreed that glucose is transported in Saccharomyces cerivisae by a carrier mediated process (see literature review), a mutation involving the membrane bound carrier would affect the ability of glucosamine to diffuse across the membrane. There is also a possibility that a mutation might change the environment of the carrier so that it does not transport as much sugar as it does in normal cells. If sugars are taken up by active transport using polyphosphates as energy (Van Steveninck, 1968), a mutation of the enzymatic reaction which forms polyphosphate could limit the transport of sugar into the cells.

On the other hand, a modification of hexokinase could limit or prevent the phosphorylation of glucosamine, also protecting mutants from depletion of inorganic phosphate or ATP in the mitochondrion.

It has been demonstrated that nuclear genes are required for the adenine nucleotide translocator (see literature review) and therefore nuclear mutations could alter the activity of the translocase. Also, both nuclear

and mitochondrial genes are required for the normal function of mitochondria; nuclear mutation could also change the activity of enzymes involved in the interconversion of adenine nucleotides. Mutation of the translocase system, that is if the substrate affinity of the adenine nucleotide translocator is changed, could slow the rate of ADP translocation into the mitochondrion and ATP out. This would limit the rate of phosphorylation via hexokinase. Mutation of the adenine nucleotide interconverting enzymes, e.g., Adenylate kinase, could limit the translocation of ADP into the mitochondria and ATP out, thus protecting mitochondria from ATP or Pi depletion.

So the final effect of any of the above mutations is the protection of mitochondrion from Pi depletion, by limiting the utilization of ATP via phosphorylation of glucosamine.

Before I start to discuss the mutants it will be better if I outline the properties of wild type yeast cells (strain 4B2) first. GR6 and $10P_3r$ are cytoplasmic mutants and very similar to each other in their properties, so I will discuss them together. GR81 and GR108, although they are nuclear mutants, differ in their properties, which is probably due to difference in the mutational site (Ball, Elliot and Wong, 1976) so these mutants will be discussed separately.

4B2 (Wild Type)

Wild type cells grow on both fermentable and non-fermentable carbon sources (Figs. 4, 5, 6, and 7). Growth rates on fermentable carbon sources, e.g., maltose and glucose, are similar (Figs. 5 and 6) but on galactose the cells show a slower growth rate (Fig. 7). This slow growth rate on galactose is due to the fact that growth on galactose is partially dependent on respiration (Ball and Tustanoff, 1971). The growth rate on non-fermentable carbon source is slower than on fermentable carbon source (Table I). All the growth rates on different media are logarithmic (Figs. 4, 5, 6, and 7).

4B2 cells transport glucose and glucosamine, which is indicated by the linear uptake of these sugars (Appendix, Figs. 1 and 2). The Lineweaver-Burk graphs indicate that sugar transport obeys Michaelis-Menton kinetics (Figs. 10 and 11). 4B2 shows a higher Vmax for glucose and glucosamine transport than the mutants (Figs. 10 and 11 and Table III) which means that wild type cells transport more sugar than mutants. The in vivo phosphorylation rate of 4B2 is higher than mutants (Table VII). Also 4B2 cells show non-linear phosphorylation, although there is linear transport of sugars, which probably indicates that sugars are taken up by both fascilitated diffusion and active transport mechanisms, depending on ATP concentration (Cirillo, 1970).

From the above experiments we can suggest the

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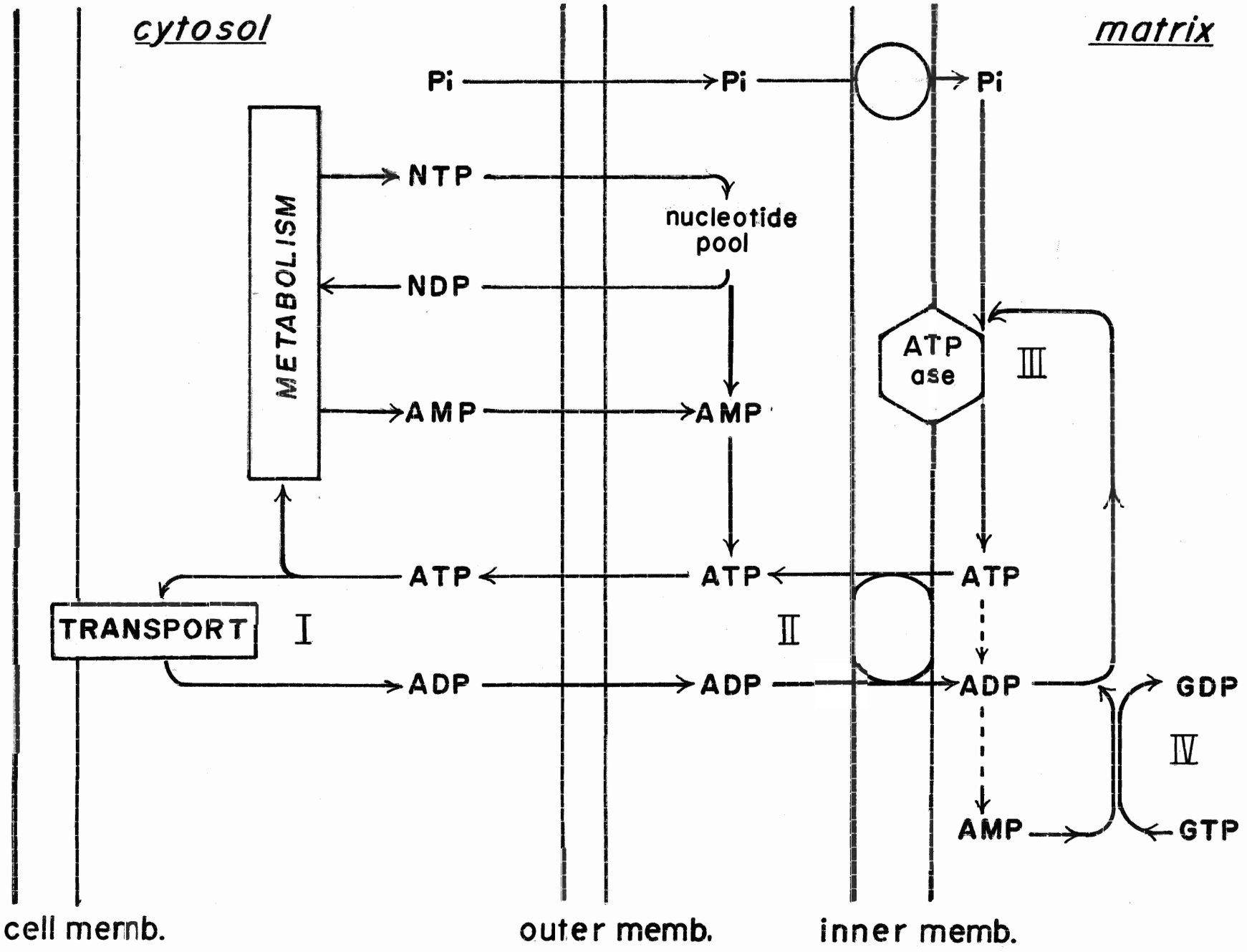
Figure 15: The Possible Role of Adenine Nucleotides in Glucosamine Poisoning.

Site I	Hexokinase
Site II	Translocase
Site III	ATP synthase
Site IV	AMP transferase


cytosol
matrix
Pi
Pi
Pi
METABOLISM
NTP
nucleotide pool
NDP
ATP ase
III
AMP
AMP
ATP
ATP
ATP
TRANSPORT
I
II
ADP
ADP
ADP
GDP
IV
AMP
GTP
cell memb.
outer memb.
inner memb.

following mechanism for respiratory inhibition in 4B2 in the presence of glucosamine. The transport of sugar utilizes ATP for phosphorylation (Fig. 15, step I) which increases the cytoplasmic ADP level which then stimulates the translocating system transporting ADP into the mitochondrion (Fig. 15, step II). This ADP stimulates ATP synthase producing ATP with utilization of inorganic phosphate (Fig. 15, Step III). A continued demand for ATP, due to high transport rate for glucosamine probably leads to more ATP formation with the subsequent depletion of inorganic phosphate within the mitochondrion. The inhibition of respiration is due to the limitation of inorganic phosphate (see Pi effects in literature review).

This is confirmed by the following experiment. When excess inorganic phosphate is added to cells in the presence of glucosamine the inhibition of respiration is not observed (Fig. 13a). The respiratory inhibition caused by Pi depletion is due to the coupled mechanisms of respiration and phosphorylation; when oligomycin (an uncoupler) is added in the presence of glucosamine, the respiratory inhibition is not observed (unpublished results by Dr. A. J. S. Ball) which confirms that respiration, phosphorylation and respiratory inhibition are connected in 4B2.

The glucosamine inhibition (of growth) in 4B2 cells is probably due to ATP depletion in the mitochondrion.

Subik, Kolarov and Kovac (1974) showed that in the presence of bongkrekic acid (BA, an inhibitor of translocase) and antimycin A (inhibitor of respiration) glucose grown wild type cells were converted to respiration deficient mutants and growth was arrested. They suggested that yeast cells cannot multiply if ATP is not available in the mitochondrion. Glucosamine poisoning would accomplish this in YPG grown cells.

From the above discussion it is clear that mutation of any of the enzymes (i) - (iv) mentioned above could be involved in relief of glucosamine poisoning in 4B2.

GR6 and $10P_3r$ (Cytoplasmic Mutants GGM-r)

Preliminary experiments, that is the growth on YPG and YPD media (Figs. 4 and 5) and respiratory ability on YPG and YPD media (Figs. 8 and 9), indicate that these two mutants are very similar to 4B2 and suggest that these mutants have normal glycolytic and respiratory enzymes.

The respiration of YPG grown cells (GR6 and $10P_3r$) increases in the presence of added inorganic phosphate (Figs. 13d and e) which indicates that oxidation and phosphorylation are coupled in their mitochondria and also that respiration is Pi limited.

The glucose, glucosamine and sorbose transport experiments for these mutants indicate that the carrier

affinity for glucose, glucosamine and sorbose is unaltered (Table III and Figs. 10, 11, and 12). As glucose, glucosamine and sorbose share the same carrier (Cirillo, 1962; Kotyk, 1973) it is not surprising that the affinity for carrier is unchanged for all three sugars in these mutants. Both GR6 and $10P_3r$ show a lower Vmax for all sugars than 4B2. Because transport is dependent on ATP concentration inside the cells the low Vmax can be due to any of the reasons (ii) - (iv) above.

Normal respiratory ability, normal growth on YPG and normal cytochrome oxidase activity (Table VIII) indicate that the production of ATP in mitochondria is normal. The normal translocation of ATP from mitochondria to cytoplasm is indicated by the normal growth on YPG and YPD media.

The change in Vmax is probably due to the change in facilitated diffusion (Fig. 11) so that less sugar will be available inside the cell for phosphorylation. Sorbose transport, which does not involve energy metabolism, indicates a change in facilitated diffusion, which can be due to a change in the carrier itself or a change in the environment of carrier so that it does not translocate sugar as well as it does in the wild type. Strains GR6 and $10P_3r$ are probably resistant to glucosamine for the following reason:

Due to the low transport rate for sugars, less ATP will be required for phosphorylation and less ADP will be

available to mitochondria for phosphorylation via the ATPase reaction which requires inorganic phosphate. Because of lower utilization of Pi, the concentration of Pi does not go down thus protecting these mutants from glucosamine poisoning.

GR81 (Nuclear Mutant, gay1)

Preliminary experiments indicate that GR81 is very similar to 4B2 except for its growth on fermentable carbon sources. Respiratory ability on both YPG and YPD media (Figs. 8 and 9) and growth on YPG medium (Fig. 4) indicate that the respiratory pathway is normal in GR81. This is further confirmed by the normal Cytochrome oxidase activity (Table VIII). GR81 shows lower growth rates on the fermentable carbon sources glucose, maltose and galactose (Figs. 5, 6, and 7) than 4B2, which probably indicates that there is something wrong with the functioning of the glycolytic pathway. Also, when oligomycin (an uncoupler) was added to glucose grown GR81 cells, the growth rate was inhibited (unpublished results by Dr. A. J. S. Ball) indicating that the growth of GR81 on fermentable carbon source is partially dependent on respiration. This experiment further supports the idea that in GR81 the lesion is probably in or connected with the glycolytic pathway. The genetic lesion could also affect ATP conversion to other nucleotides (ADP or AMP, Fig. 15) also altering the ATP - ADP balance and therefore glycolysis (Atkinson, 1966 and 1968; Ball and

Atkinson, 1975).

The respiration of YPG grown GR81 cells increases in the presence of inorganic phosphate (Fig. 13b); also the partial inhibition by glucosamine is not observed in the presence of inorganic phosphate (Fig. 13b and Table IV). This indicates that the basic mechanisms of the Crabtree effect (as outlined in the 4B2 section) is normal in GR81, but the degree of poisoning is less.

Glucose, glucosamine and sorbose transport by GR81 (Figs. 10, 11, and 12) indicates that the carrier affinity for glucose, glucosamine and sorbose in unaltered (Km unchanged) although GR81 shows a lower Vmax for glucose and glucosamine transport (Table III and Figs. 10 and 11) while for sorbose the Vmax remains the same (Fig. 12 and Table III). This indicates that in GR81 the low Vmax for glucose and glucosamine transport is due to a change in energy metabolism rather than in translocation of sugar (cf. GR6 above). That the transport of sugar is dependent on ATP concentration has been shown in Schizosaccharomyces pombe by Foury and Goffeau (1975). They indicated that in the presence of respiratory and oxidative phosphorylation inhibitors there is a decrease in both ATP content and glucose uptake (see also sugar transport in Literature Review).

The ATP utilization during transport could be due to hexokinase activity (Cirillo, 1970) or phosphorylation

associated with transport (Van Steveninck, 1968). The *in vitro* hexokinase activity is normal (Table V and VI and Figs. 14a and b) which indicates that phosphorylation via hexokinase should be normal. The *in vivo* phosphorylation is lower (Table VII) which indicates that GR81 utilizes less ATP than 4B2 and facilitated diffusion is the dominant form of transport in GR81. The low utilization of ATP could be due to (i) low ATP concentration in the cytosolic compartment (Cirillo, 1970; Kotyk, 1973; Foury and Goffeau, 1975) or (ii) low amount of carrier phosphate complex which could be due to a low ATP concentration or to the mutation of the enzyme(s) involved in the formation of polyphosphates (Van Steveninck, 1968). There is not enough evidence to clearly choose between these two versions of sugar transport.

Galactosamine, which is taken up by yeast cells on a different carrier than glucose or glucosamine (Cirillo, 1970; Kotyk, 1973) also inhibits respiration in galactose grown 4B2 (unpublished results). However GR81 is resistant to galactosamine poisoning suggesting that carrier for transport of glucose and glucosamine are normal but the availability of ATP for active transport, which is common in glucosamine and galactose metabolism, is altered.

The slow growth on YPD could be due to altered utilization of ATP during transport because glycolysis usually keeps the ATP concentration high (Lehninger, 1975).

A dominance of facilitated diffusion (Fig. 3) could keep the ATP concentration high in glucose grown cells and a high concentration of ATP could allosterically inhibit phosphofructokinase activity (Passonneau and Lowry, 1964) which would ultimately inhibit the glycolytic pathway. This would also explain the dependence on respiration during growth on fermentable carbon source (oligomycin experiment). As the growth on fermentable carbon source, e.g., glucose, is dependent on the transport of glucose (Polakis and Bartley, 1964 and 1965), the slow growth rate on YPD is probably due to slow transport of glucose.

Unlike 4B2, GR81 is partially derepressed in YPD as judged by the gradual increase in QO_2 (Fig. 9), i.e., when glucose utilization is slowed down (because of transport) mitochondriogenesis is partially derepressed. This has been hypothesised before (see literature review) but does not help to explain the Crabtree effect or glucose repression. GR81, unlike 4B2, remains partially derepressed throughout the growth on YPD medium and a more detailed analysis of GR81 might help to discover how glucose repression works in yeast cells.

GR81 is resistant to glucosamine poisoning due to less utilization of ATP (due to the dominance of facilitated diffusion) during sugar transport making less ADP available for phosphorylation via the ATP ase reaction which requires inorganic phosphate (Pi). Because of lower utilization of Pi, the concentration of Pi does not

go down thus protecting GR81 cells from glucosamine poisoning.

GR108 (Nuclear Mutant, gav2)

The preliminary experiments show a slow growth rate for GR108 on YPG medium (Fig. 4) which means that GR108 probably has less mitochondria than 4B2, as growth on glycerol is dependent on mitochondria, (Figs. 4 and 8). GR108 grows normally on fermentable carbon sources glucose and maltose (Figs. 4 and 6) but on galactose it again grows more slowly (Fig. 7). The slow growth on galactose is probably due to the low QO_2 (Figs. 8 and 9). GR108 also exhibits low respiratory ability when growing on both YPG and YPD media (Figs. 8 and 9) and low cytochrome oxidase activity (Table VIII) which further confirms that GR108 has a lesion which affects mitochondriogenesis.

The respiration of YPG grown cells increases slightly in the presence of added inorganic phosphate (Fig. 13c) which suggests that the basic mechanism, that is the oxidation and phosphorylation reactions are coupled in the mitochondrion. The slight inhibition by glucosamine (Fig. 13c) is not observed in the presence of inorganic phosphate which again indicates that glucosamine inhibition is due to the lowering of inorganic phosphate concentration in mitochondria (cf 4B2 above).

Glucose, glucosamine and sorbose transport studies on GR108 indicate that the carrier affinity for glucose,

glucosamine and sorbose is unaltered in GR108 (Table III). GR108 shows a lower Vmax for glucose and glucosamine transport while the Vmax for sorbose remains unchanged (Figs. 10, 11, and 12 and Table III). This indicates that in GR108 the low Vmax for glucose and glucosamine is due to a change in energy related metabolism rather than in translocation of sugar (cf. GR6 and GR81 above). This is confirmed by the resistance of GR108 to galactosamine poisoning (cf. GR81 above). The low *in vivo* phosphorylation rate (Table VII) further confirms that the low transport rate is due to changes in energy metabolism. The low phosphorylation rate could be due to the mutation of any of the above mentioned biochemical reactions (ii) to (iv). Step (ii) is unlikely as glucosamine kinase is normal (Table VI).

GR108 grows well on fermentable carbon source and also the growth of GR108 on YPD in the presence of oligomycin remains unaltered (unpublished results by Dr. A. J. S. Ball). This indicates that the ATP-ADP translocase is probably normal (Subik *et al*, 1974). The most likely reasons for the low production of ATP on YPG medium are:

(i) Altered AMP transferase activity (Fig. 15, Step IV) so that ADP inside the mitochondria is converted to AMP and less ADP is available for phosphorylation through ATP synthase activity. This will also limit ATP$\rightleftharpoons$ADP

exchange between the mitochondrion and the cytosol (Klingenberg, 1976).

(ii) Any one of the enzymes of the respiratory chain might be affected, e.g., cytochrome oxidase.

Our experiments indicate that GR108 has low cytochrome oxidase activity and a low QO_2 which could be due to the low ATP production (see (i) above) or cause the low ATP production in the mitochondria. If the biosynthesis of mitochondria was affected by the mutation there would be less mitochondria, but they would be sensitive to inhibition, unless the mutation which prevented biosynthesis was also involved in the glucosamine effect. From these results we can suggest the following mechanism of glucosamine resistance in GR108.

Due to the low rate of ATP production in the mitochondrion inorganic phosphate concentration is not limiting (Fig. 13c). As sugar transport is dependent on ATP concentration, low ATP concentration lowers the transport of sugar with less production of ADP. So in GR108 the lesion, which causes low production of ATP, protectes these cells from glucosamine poisoning because inorganic phosphate is only utilized slowly in the mitochondrion. The lesion may be in a mitochondrial enzyme.

GR108 also shows repression of respiration by glucose and on exhaustion of glucose, cells are sharply derepressed (Fig. 9). The repression and derepression of mitochondriogenesis is similar to 4B2 (Fig. 9). Because

of its similarity to 4B2, GR108 would not be suitable for studying glucose repression although this mutant might be useful for studying the Crabtree effect.

Conclusion

The experiments have not revealed the exact lesions that cause glucosamine resistance in these four strains. The mutation in GR6 and $10P_3r$ probably causes altered membrane properties which reduces facilitated diffusion as indicated by normal *in vitro* hexokinase activity but slow transport of glucose, glucosamine and sorbose. There is not enough evidence to explain the slow transport in these two strains.

The mutation *gay*^{1} (GR81) in some way affects the availability or utilization ot ATP for transport as energised transport is reduced in this mutant as indicated by both *in vivo* phosphorylation and sorbose transport experiments. The evidence does not explain the lowered availability or utilization of ATP in GR81 mutant.

The mutation *gay*^{2} (GR108) influences the generation of ATP via oxidative phosphorylation, which may be related to a reduced cytochrome oxidase activity in this mutant.

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APPENDIX

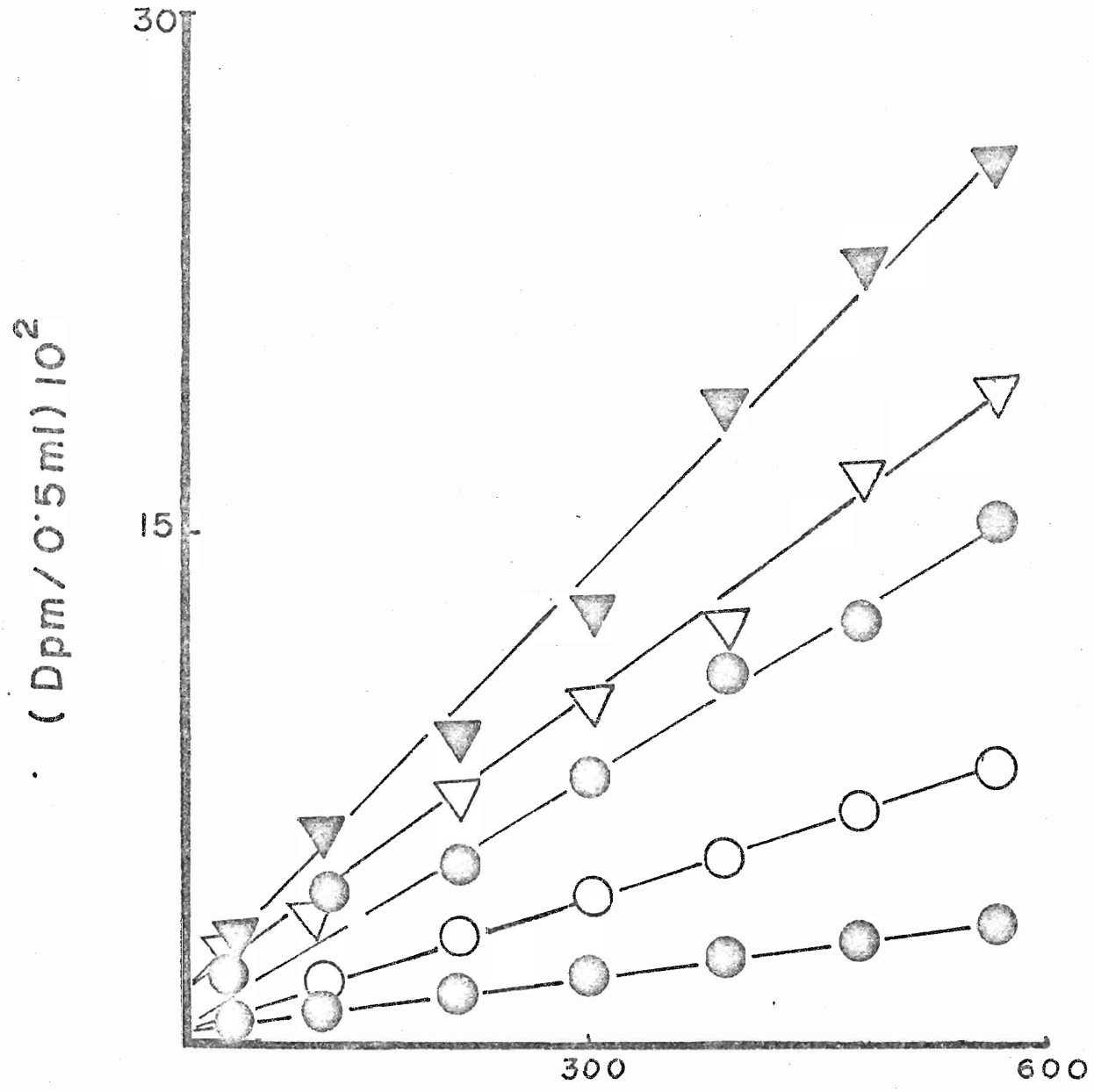
LEGEND

Appendix Figure 1: Glucose Transport.

Cells were grown to mid log phase on YPG and glucose transport was done on unwashed whole cells as described in the Methods.

The curves indicate glucose concentration from low to high range.

GR 108 GLUCOSE UPTAKE



LEGEND

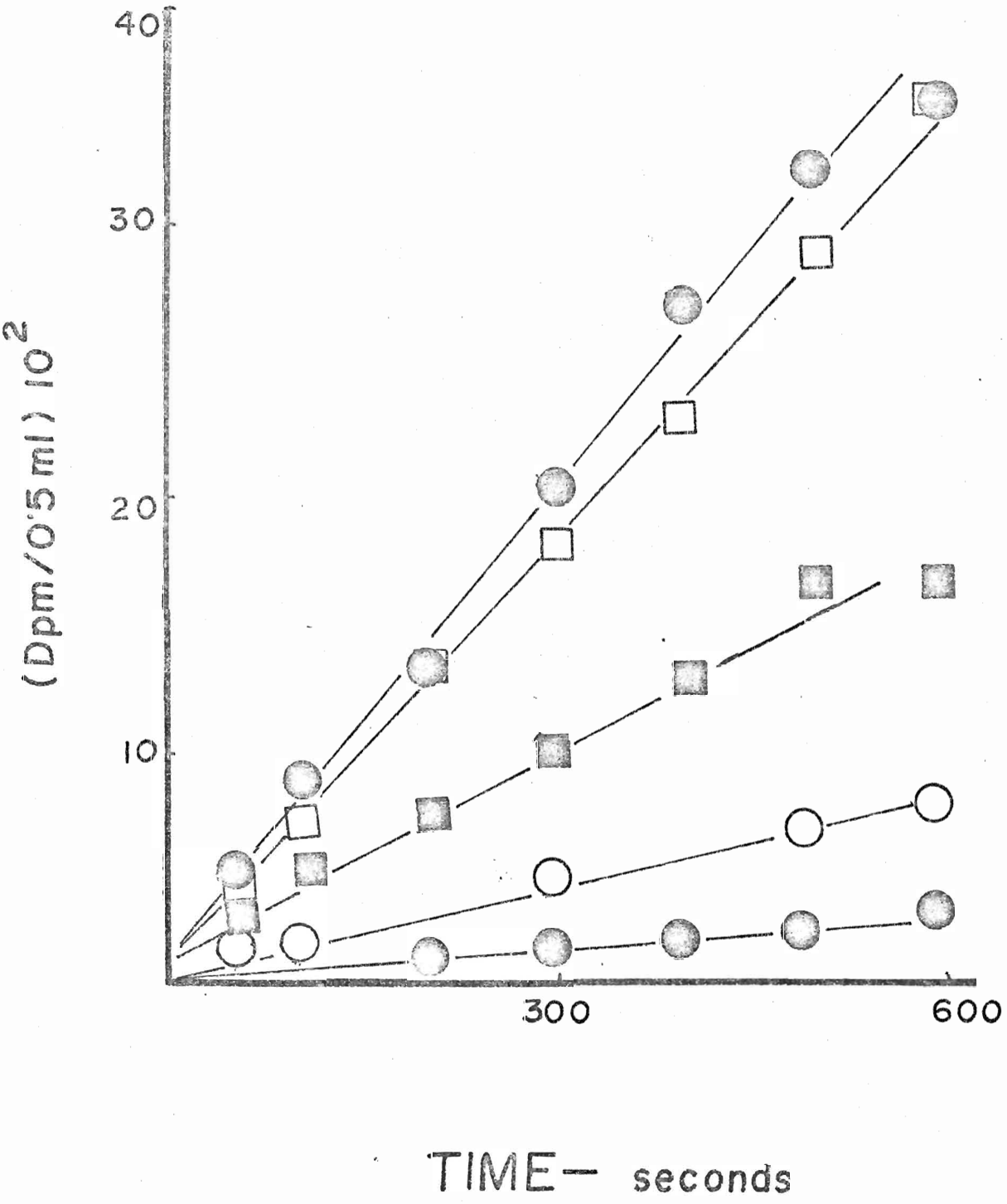
Appendix Figure 2: Glucosamine Transport.

Cells were grown to mid log phase on YPG and glucosamine transport was done on unwashed whole cells as described in the Methods.

The curves indicate glucosamine concentration from low to high range.

4B2

GLUCOSAMINE UPTAKE



LEGEND

Appendix Figure 3: In Vitro Phosphorylation or Glucosamine Kinase.

Activities were determined on French-pressed cell free extracts of cells grown to mid log phase in YPG. Activity was determined by using ^{14}C assay as specified in the methods.

4B2 GLUCOSAMINE KINASE